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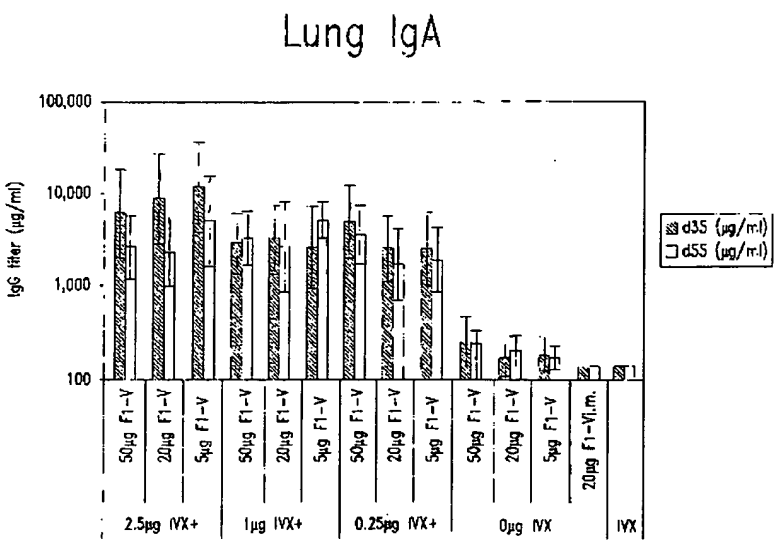

(57) Abstract: Methods for making and using therapeutic formulations of Proteosome-based immunoactive compositions are provided.

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PROTEOSOME-BASED FOR VACCINATING AGAINST INFECTIOUS DISEASES

TECHNICAL FIELD

This disclosure relates generally to nasally-delivered vaccines for protecting against infectious disease or toxic agents and, in particular, to Proteosome-based compositions and methods of use thereof for treating or preventing various diseases associated with microbial pathogens or toxic agents, such as *Yersinia pestis*, Variola virus, ricin toxin and botulinum toxin.

BACKGROUND

An efficient immune response depends on the communication between the innate immune system (the first line of defense against invading pathogens) and the adaptive (specific) immune response (*see generally*, Klein *et al.*, Immunology (2nd), Blackwell Science Inc., Boston, 1997). T lymphocytes are important for coordinating the adaptive immune response by controlling the release of effector molecules. For example, T helper (Th) 1 cells produce interleukin-2 (IL-2), tumor necrosis factor alpha (TNF-α), and interferon gamma (IFN-γ), which are important for the development of cell-mediated immunity (Mosmann *et al.*, *J. Immunol. 136*: 2348, 1986; Street and Mosmann, *FASEB J. 5*: 171, 1991). In contrast, Th2 cells produce IL-4, IL-13, IL-5, IL-9, IL-6 and IL-10, which are important for the stimulation of IgE production, mucosal mastocytosis, and eosinophilia (Mosmann *et al.*; Street and Mosmann). While a shift toward a Th1 (type 1 response) or Th2 (type 2 response) phenotype may be important for the defense against pathogens, a shift in one direction or another can also be associated with the induction of autoimmune disease (Th1) or inflammatory disease (Th2).

In this regard, vaccines have been developed and used to induce an adaptive immune response. Vaccines typically include an attenuated microbe or a microbial antigen (*i.e.*, a microbe component such as a protein or nucleic acid) to activate a specific immune response. However, the ability of an antigen to induce a protective immune response in a host sometimes must be enhanced by formulating the

antigen with an immunostimulant or an adjuvant. For over 100 years, aluminum-based mineral salts (generically known as alum) have been the only adjuvants approved for human use. Historically, alum has been an effective adjuvant by significantly enhancing responses to adsorbed proteins when injected intramuscularly. However, in recent years, alum adjuvants has come under scrutiny due to reports (1) that alum is a weak adjuvant for the new generation of vaccine antigens, which are based on highly purified recombinant proteins; (2) that alum can reportedly induce IgE antibodies and has been associated with allergic reactions in some vaccine recipients; and (3) that alum can induce persistent inflammatory responses which are manifested as an adverse reaction at injection sites. These adverse reactions have been found to be associated with the tendency of alum-adsorbed vaccines to polarize antigen-specific immune responses towards a type 2 phenotype (*i.e.*, inflammatory response). Furthermore, alum-based vaccines have only been approved for use in humans by the injectable route.

Other known adjuvants include Freund's adjuvant (complete or incomplete), lipopolysaccharide (LPS, also referred to as endotoxin), and pertussis adjuvant (a saline suspension of killed *Bordatella pertussis* organisms). Freund's adjuvant consists of a mixture of mycobacteria in an oil/water emulsion. However, due to frequent toxic physiological and immunological reactions to this material, Freund's adjuvant cannot be used in humans. LPS stimulates the immune system by triggering an innate immune response, but LPS is too toxic to be a viable adjuvant. Molecules that are structurally related to endotoxin, such as monophosphoryl lipid A ("MPL" or detoxified LPS), are being tested as adjuvants in clinical trials. Other potential adjuvants being investigated include saponins (*see* U.S. Patent Nos. 6,080,725; 6,262,029; and 5,977,081); amphipathic aldehydes (*see* U.S. Patent No. 6,649,172); proteosomes (*see* U.S. Patent Nos. 5,726,292 and 5,985,284; U.S. Patent Application Nos. 2001/0053368 and 2003/0044425); and cyclic aminoalkyl glucosaminide phosphates (*see* U.S. Patent No. 6,525,028).

Hence, there is a recognized need in the art for identifying and developing superior immunostimulatory compounds that can be co-administered with

antigens to promote more robust immune responses to protect against microbial infections or toxic agents. The present invention meets such needs, and further provides other related advantages.

BRIEF SUMMARY OF THE INVENTION

The present disclosure provides adjuvant formulations for antigens, in particular Proteosome-based adjuvants for formulation with various antigens for use in a variety of therapeutic settings, such as in treating or preventing, for example, infectious disease or the action of toxic agents.

In one aspect, the present invention provides a method for eliciting an immune response to an antigen, comprising administering to a subject in need thereof a therapeutically effective amount of an immunogenic composition, wherein said immunogenic composition comprises Proteosomes, liposaccharide and an antigen. In another aspect, the present invention provides a method for treating or preventing a microbial infection, comprising administering to a subject in need thereof a therapeutically effective amount of an immunogenic composition, wherein said immunogenic composition comprises Proteosomes, liposaccharide and an antigen. In certain embodiments, the antigen of the immunogenic compositions comprises one or more from *Bacillus anthracis, Chlamydia trachomatis, Staphylococcus aureus, Clostridium perfringens,* or *Yersinia pestis*. In certain other embodiments, the antigen of the immunogenic compositions comprises one or more from Poxvirus, Alphavirus, Flavivirus, Arenavirus, Bunyavirus, Filovirus, or Picornavirus. In yet other embodiments, the antigen of the immunogenic compositions comprises one or more toxins. In still other embodiments, the antigen is recombinant. In yet other embodiments, the immunogenic composition comprises a plurality of different antigens. In a related embodiment, the plurality of antigens can comprise viral, bacterial, parasitic, toxin, or a combination thereof.

In certain embodiments, the immunogenic composition is administered by a route selected from mucosal, enteral, parenteral, transdermal, transmucosal, nasal, or inhalation, or a combination thereof. In a related embodiment, the immunogenic

composition elicits a protective immune response. In other embodiments, the antigen comprises a Protective Antigen from *Bacillus anthracis*, filamentous hemagglutinin (FHA) of *Boretella pertussis*, an F1 antigen or a V antigen from *Yersinia pestis*, an F1-V antigen fusion protein antigen, or a combination thereof. In still other embodiments, the antigen comprises one or more antigens from a Variola virus or from a Vaccinia virus. . In yet other embodiments, the antigen comprises one or more of botulinum toxin, ricin toxin, Staphylococcus enterotoxin B, or fragment or variant thereof.

In still other embodiments, any of the aforementioned immunogenic compositions have a liposaccharide final content by weight as a percentage of Proteosome protein ranges from about 1% to about 500% in the immunogenic composition. In other embodiments, the Proteosomes and liposaccharide are obtained from the same bacteria or from different bacteria, such as Gram-negative bacteria. In some embodiments, the liposaccharide is from Gram-negative bacteria selected from *Shigella* species, *Chlamydia* species, *Yersinia* species, *Pseudomonas* species, *Plesiomonas* species, *Escherichia* species, *Salmonella* species, or a combination thereof. In other embodiments, the Proteosomes are from *Neisseria* species. In certain embodiments, the immunogenic compositions of this disclosure have Proteosomes are from *Neisseria meningitides* and the liposaccharide is from *Shigella flexneri*. In other embodiments, any of the aforementioned immunogenic compositions have a ratio of Proteosomes to antigen that ranges from about 5:1 to about 1:500, or the ratio is at least 1:2, 1:5, or 1:10. In still other embodiments, any of the aforementioned immunogenic compositions further comprise a pharmaceutically acceptable carrier, excipient or diluent.

In another aspect, the instant disclosure provides a method for eliciting an immune response to an antigen, comprising administering to a subject in need thereof a therapeutically effective amount of an immunogenic composition, wherein said immunogenic composition comprises an adjuvant and an antigen, wherein said adjuvant comprises Proteosomes and liposaccharide and said adjuvant to antigen ratio ranges from about 1:5 to about 1:500 or is at least 1:5, such that an immune response to said antigen is elicited. In a related aspect, there is provided a method for treating or

preventing a microbial infection, comprising administering to a subject in need thereof a therapeutically effective amount of an immunogenic composition, wherein said immunogenic composition comprises an adjuvant and an antigen, wherein said adjuvant comprises Proteosomes and liposaccharide and said adjuvant to antigen ratio ranges from about 1:5 to about 1:500 or is at least 1:5, such that a microbial infection is treated or prevented. In certain embodiments, provided is any of the aforementioned immunogenic compositions wherein the antigen is at least one of a bacterial, viral, or toxin antigen.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth herein which describe in more detail certain procedures or compositions, and are therefore incorporated by reference in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows serum IgG titers from mice immunized twice intranasally with 50, 20 or 5μg of F1-V with or without Protollin (2.5, 1 or 0.25 μg), or injected intramuscularly with 20μg F1-V adsorbed onto alum (Alhydrogel®). Half the mice were euthanized on day 35 post-primary immunization, and the other half on day 55. Titers are expressed as the geometric mean of specific antibody concentrations (μg/ml for serum IgG; ng/ml for lung IgA and IgG) and 95% confidence limits are shown.

Figure 2 shows lung IgG titers from mice immunized as described for Figure 1.

Figure 3 shows lung IgA titers from mice immunized as described for Figure 1.

Figures 4A and 4B show the survival of mice against challenge with lethal doses of aersolized *Yersinia pestis*. Mice immunized twice with 20 μg F1-V intranasally with (0.25, 1, or 2.5 μg) or without Protollin, or intramuscularly adsorbed onto Alhydrogel®, were challenged by whole body exposure to 170 LD_{50} of *Y. pestis* (A) 35 days or (B) 55 days post-primary immunization. In all studies, all control mice

(that received Protollin only) died when challenged with *Y. pestis*, confirming the inoculum used was lethal.

Figures 5A and 5B show the survival of mice against challenge with lethal doses of aersolized *Yerstnia pestis*. Mice immunized twice with 50 μg F1-V intranasally with or without 1 μg of Protollin, or intramuscularly adsorbed onto Alhydrogel®, were challenged by whole body exposure to (A) 255 LD_{50} of *Y. pestis* 55 days post primary immunization; (B) shows the survival of mice challenged on day 35 and day 55 (different groups of mice) post-primary immunization with 170 lethal doses of aersolized *Y. pestis*. Mice were immunized twice with 5 μg F1-V intranasally with (0.25, 1, or 2.5 μg) or without Protollin. All the control mice (those that received Protollin only) died when challenged with *Y. pestis*, confirming the inoculum used was lethal.

Figure 6 shows the amounts of IFN-γ, TNF-α and IL-5 released from splenocytes stimulated *in vitro* with antigen F1-V. Splenocytes were harvested from mice immunized intranasally with 50 μg of F1-V with or without 1 μg Protollin, or injected intramuscularly with 20 μg of F1-V adsorbed onto Alhydrogel®, on day 35 after immunization.

Figures 7A and 7B show serum IgG and lung IgA levels, respectively, in mice immunized nasally (on days 0 and 14) with 5 μg or 25 μg of recombinant Protective Antigen (rPA) from *Bacillus anthracis* admixed with (1 μg) or without Protollin.

Figures 8A and 8B show results of anthrax neutralization assays using serum and lung lavage fluid from mice immunized with rPA admixed with Protollin.

Figure 9 shows serum IgG in mice immunized (on days 1, 21 and 31) with 15 μg of recombinant filamentous hemagglutinin (rFHA) from *Bordetella pertusis* admixed with liposome (intranasally, i.n.), Protollin (i.n.), or Alhydrogel® (intraperitoneally, i.p.). As controls, mice were immunized i.p. with Quadracel™ (positive) and i.n. with excipient PBS alone (negative). Quadracel™ (Aventis Pasteur Ltd.) is a vaccine containing, among other antigens, *Bordetella pertusis* FHA. Titers were determined by measuring the level of anti-rFHA in serum harvested seven days

after the last immunization (day 38) (less the background titer level found, if any, in serum from mice on day 1).

Figure 10 shows salivary IgA in mice immunized (on days 1, 21 and 31) with 15 μg of recombinant filamentous hemagglutinin (rFHA) from *Bordetella pertusis* formulated in the same compositions as described for Figure 9.

Figure 11 shows anti-rFHA serum IgG subclasses (IgG2a and IgG1) found in pooled sera from mice immunized as described for Figure 9.

Figure 12 shows the level of *Bordetella pertusis* found in the lungs of immunized mice as described for Figure 9 when challenged with aerosolized *B. pertusis* eleven days after the final immunization (day 42). Lung counts were measured on days 1, 3, 7, and 14 post-aerosol challenge.

DETAILED DESCRIPTION OF THE INVENTION

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms that will be used hereinafter.

"Proteosome or Projuvant" as used herein refers to preparations of outer membrane proteins (OMPs, also known as porins) from Gram-negative bacteria, such as Neisseria species (*see, e.g.*, Lowell *et al.*, *J. Exp. Med. 167*:658, 1988; Lowell *et al.*, *Science 240*:800, 1988; Lynch *et al.*, *Biophys. J. 45*:104, 1984; Lowell, in "New Generation Vaccines" 2nd ed., Marcel Dekker, Inc., New York, Basil, Hong Kong, page 193, 1997; U.S. Patent No. 5,726,292; U.S. Patent No. 4,707,543), which are useful as a carrier or an adjuvant for immunogens, such as bacterial or viral antigens. Proteosomes are hydrophobic and safe for human use, and comparable in size to certain viruses. Proteosomes have the interesting ability to auto-assemble into vesicle or vesicle-like OMP clusters of 20-800 nm, and to noncovalently incorporate, coordinate, associate (*e.g.*, electrostatically or hydrophobically), or otherwise cooperate with protein antigens (Ags), particularly antigens that have a hydrophobic moiety. Any preparation method that results in the outer membrane protein component in vesicular or vesicle-like form, including multi-molecular membranous structures or molten globular-like OMP compositions of one or more OMPs, is included within the definition of Proteosome.

Proteosomes may be prepared, for example, as described in the art (*see, e.g.*, U.S. Patent Nos. 5,726,292 or 5,985,284).

"Liposaccharide" as used herein refers to native (isolated or prepared synthetically with a native structure) or modified lipopolysaccharide or lipooligosaccharide (collectively, also referred to as "LPS") derived from Gram-negative bacteria, such as *Shigella flexneri* or *Plesiomonas shigelloides*, or other Gram-negative bacteria (including *Alcaligenes, Bacteroides, Bordetella, Borrellia, Brucella, Campylobacter, Chlamydia, Citrobacter, Edwardsiella, Ehrlicha, Enterobacter, Escherichia, Francisella, Fusobacterium, Gardnerella, Hemophillus, Helicobacter, Klebsiella, Legionella, Leptospira* (including *Leptospira interrogans*), *Moraxella, Morganella, Neiserria, Pasteurella, Proteus, Providencia*, other *Plesiomonas, Porphyromonas* (including *Porphyromonas gingivalis*), *Prevotella, Pseudomonas, Rickettsia, Salmonella, Serratia,* other *Shigella, Spirillum, Veillonella, Vibrio*, or *Yersinia* species). The liposaccharide may be in a detoxified form (*i.e.*, having the Lipid A core removed) or may be in a form that has not been detoxified. In the instant disclosure, liposaccharide need not be and preferably are not detoxified. The liposaccharide may be prepared, for example, as described in U.S. Patent Application Publication No. 2003/0044425.

"Proteosome:LPS or Protollin or IVX or IVX-908" as used herein refers to preparations of projuvant admixed as described herein with at least one kind of liposaccharide to provide an OMP-LPS composition (which can function as an immunostimulatory composition). Thus, the OMP-LPS adjuvant can be comprised of two of the basic components of Protollin, which include (1) an outer membrane protein preparation of Proteosomes (*i.e.*, Projuvant) prepared from Gram-negative bacteria, such as *Neisseria meningitides*, and (2) a preparation of one or more liposaccharides. Protollin should also be understood to optionally include lipids, glycolipids, glycoproteins, small molecules, or the like, and combinations thereof. The Protollin may be prepared, for example, as described in U.S. Patent Application Publication No. 2003/0044425.

Projuvant is generally used in conjunction with antigens that possess a (naturally-occurring or modified) hydrophobic moiety (also referred to as a "foot" or "anchor"). Protollin (with exogenously added LPS), which although can be used with an antigen that do not contain a hydrophobic foot domain and which are largely hydrophilic in nature, or can be associated with a antigen containing a hydrophobic foot, or a combination thereof.

"Toxin or Toxic Agent" as used herein refers to is a noxious or poisonous substance that is antigenic and is formed or elaborated as an integral part of a cell or tissue (*e.g.*, endotoxin), as an extracellular product (*e.g.*, exotoxin), or as a combination of the two, during the metabolism or growth of certain microorganisms or some higher plant and animal species. For use in the immunogenic compositions of the instant disclosure, it should be understood that a toxin or toxic agent will be in the form of a "toxoid" so that the immunogenic compositions are not toxic or poisonous. A "toxoid" form is a toxin that has been treated so as to destroy the toxic property of the agent while still retaining antigenicity (*i.e.*, capable of eliciting anti-toxin or a neutralizing immune response). The "treatment" of a toxin to generate a toxoid includes chemical treatment (*e.g.*, formaldehyde), mutations, fragments, and combinations thereof. Exemplary toxins include ricin toxin, botulinum toxin, alfatoxin, episolntoxin, Staphylococcus enterotoxin B, tetrodotoxin, snae venom toxin, diptheria toxin, cholera toxin, saxitoxin, trichothecene mycotoxins, etc.

"Immunogenic composition" as used herein refers to any one or more compounds or agents capable of priming, potentiating, activating, stimulating, augmenting, boosting, amplifying, or enhancing an adaptive (specific) immune response, which may be cellular (T cell) or humoral (B cell), or a combination thereof. Preferably, the adaptive immune response will be protective, neutralizing, or both. A representative example of an immunogen is a microbial antigen (such as one or more bacterial, viral, or parasite proteins of interest) or a non-microbial antigen (*e.g.*, antigens obtained from other sources, such as from plants (ricin), from dinoflagellates (saxitoxin), and the like).

"Specific or Acquired or Adaptive immune response" as used herein refers to a resistance mediated by a mammalian immune system resulting from previous exposure to an infectious agent or antigen. For example, specific immunity can be a result of a naturally acquired (patent or latent) infection or from an intentional vaccination. In addition, specific immunity may be passively and transitorily acquired from the transfer of antibodies from another naturally (*e.g.*, maternally inherited), or from transfer of antibodies or immune cells by intentional inoculation (*e.g.*, antibody mediated immunotherapy).

In the present description, any concentration range, percentage range, ratio range or other integer range is to be understood to include the value of any integer within the recited range and, when appropriate, fractions thereof (such as one tenth and one hundredth of an integer), unless otherwise indicated. As used herein, "about" or "comprising essentially of" mean ± 15%. The use of the alternative (*e.g.*, "or") should be understood to mean one, both, or any combination thereof of the alternatives. As used herein, the use of an indefinite article, such as "a" or "an", should be understood to refer to the singular and the plural of a noun or noun phrase. In addition, it should be understood that the individual compositions, formulations, or compounds, or groups of compositions, formulations, or compounds, derived from the various components or combinations of the composition or sequences, structures, and substituents described herein, are disclosed by the present application to the same extent as if each composition or compound or group of compositions or compounds was set forth individually. Thus, selection of particular sequences, structures, or substituents is within the scope of the present invention.

As set forth above and described herein, the present invention provides compositions and methods for using and making immunogenic compositions to treat or prevent a variety of infectious diseases or to treat or prevent pathology associated with toxic agents. In particular, provided are immunogenic compositions comprising an adjuvant (such as Proteosomes or Proteosomes with liposaccharide) and an antigen (such as microbial antigens or toxins) formulated with, for example, limited amounts of adjuvant and excess amounts of antigen. The instant invention, therefore, relates

generally to the surprising discovery that Proteosome-based adjuvants have such robust immunostimulatory activity that more antigen can be formulated with less adjuvant to provide new and useful vaccine formulations. Discussed in more detail below are immunogenic compositions comprising Proteosome:LPS or Proteosomes formulated with one or more microbial antigens. In certain aspects, the compositions of the instant description are suitable for therapeutic uses such as treating or preventing a microbial infection by inducing a specific immune response, or eliciting an immune response capable of providing protective immunity (against infection) or neutralizing immunity (against toxic agents).

PROTEOSOME-BASED VACCINE ADJUVANTS – ("PROJUVANT" AND "PROTOLLIN")

The invention relates to immunogenic compositions that contain one or more antigen capable of eliciting an immune response. As set forth above, many antigens are poorly immunogenic unless formulated with an adjuvant. The only adjuvant licensed for use in humans is alum, but this adjuvant has limitations as described above. Despite the multiplicity of efforts to identify alternative adjuvants, there remains a need for effective compositions to immunize individuals in need thereof, particularly against infectious diseases and toxic agents.

The Proteosome-based adjuvant of the instant disclosure can be used in vaccine formulations, which may include a variety of antigen sources. For example, live attenuated microbes, killed microbes, split antigens, subunit antigens, toxic agent antigens, and combinations thereof. To maximize the effectiveness of a subunit vaccines, the antigens should be combined with a potent immunostimulant or adjuvant. Exemplary adjuvants include alum (aluminum hydroxide, REHYDRAGEL®), aluminum phosphate, Proteosome adjuvant (*see, e.g.*, U.S. Patent Nos. 5,726,292 and 5,985,284, and U.S. Patent Application Publication Nos. 2001/0053368 and 2003/0044425), virosomes, liposomes with and without Lipid A, Detox (Ribi/Corixa), MF59, or other oil and water emulsions type adjuvants, such as nanoemulsions (*see, e.g.*, U.S. Patent No. 5,716,637) or submicron emulsions (*see, e.g.*, U.S. Patent No.

5,961,970), and Freund's complete and incomplete adjuvant. A particularly preferred adjuvant is a Proteosome or Protollin.

Proteosomes are comprised of outer membrane proteins (OMP) from *Neisseria* species typically, but can be derived from other Gram-negative bacteria (*see, e.g.*, Lowell *et al.*, *J. Exp. Med. 167*:658, 1988; Lowell *et al.*, *Science 240*:800, 1988; Lynch *et al.*, *Biophys. J. 45*:104, 1984; U.S. Patent No. 5,726,292; U.S. Patent No. 4,707,543). Proteosomes have the interesting ability to auto-assemble into vesicle or vesicle-like OMP clusters of 20-800 nm, and to noncovalently incorporate, coordinate, associate, or otherwise cooperate with protein antigens (Ag), particularly antigens that have a hydrophobic moiety. Proteosomes are hydrophobic and safe for human use, and comparable in size to certain viruses. By way of background, and not wishing to be bound by theory, mixing of Proteosomes with a protein (*e.g.*, antigen) provides a composition comprising non-covalent association or coordination between the antigen and Proteosomes, which association or coordination forms when solubilizing detergent is selectively removed or reduced, for example, by dialysis. Proteosomes may be prepared as described in the art or as described in U.S. Patent Application Nos. 2001/0053368 and 2003/0044425.

Any preparation method that results in the outer membrane protein component in vesicular or vesicle-like form, including molten globular-like OMP compositions of one or more OMP, is included within the definition of "Proteosome." In one embodiment, the Proteosomes are from *Neisseria* species, and more preferably from *Neisseria meningitides*. In certain embodiments, Proteosomes are not a carrier but are an adjuvant. In certain other embodiments, Proteosomes may be an adjuvant and an antigen delivery composition. In a preferred embodiment, an immunogenic composition comprises one or more antigens (*e.g.*, bacterial, viral, parasitic, fungal, toxin antigens, and fragments or variants thereof) as described herein and an adjuvant, wherein the adjuvant comprises Projuvant or Protollin. As described herein, the antigens can be from a recombinant source or comprise, for example, a (detergent) split antigen.

In certain embodiments, the invention provides an immunogenic composition that further comprises an immunostimulant, such as a liposaccharide. That is, the adjuvant may be prepared to include an additional immunostimulant. For example, the projuvant may be mixed with a liposaccharide to provide an OMP-LPS adjuvant. Thus, the OMP-LPS (Protollin) adjuvant can be comprised of two basic components. The first component is an outer membrane protein preparation of Proteosomes (*i.e.*, Projuvant) prepared from Gram-negative bacteria, such as *Neisseria meningitidis*. The second component is a preparation of liposaccharide. The liposaccharide may be prepared as described in U.S. Patent Application Nos. 2001/0053368 and 2003/0044425. It is also contemplated that the second component may include lipids, glycolipids, glycoproteins, small molecules or the like, and combinations thereof.

As described herein, the two components of an OMP-LPS adjuvant may be formulated at specific initial ratios to optimize interaction between the components resulting in stable association and formulation of the components for use in the preparation of an immunogenic composition of the invention. The process generally involves the mixing of components in a selected detergent solution (*e.g.*, Empigen® BB, Triton® X-100, or Mega-10) and then effecting complexing of the OMP and LPS components while reducing the amount of detergent to a predetermined, preferred concentration, by dialysis or, preferably, by diafiltration/ultrafiltration methodologies. Mixing, co-precipitation, or lyophilization of the two components may also be used to effect an adequate and stable association or formulation. In a preferred embodiment, an immunogenic composition comprises one or more antigens as described herein and an adjuvant, wherein the adjuvant comprises a Projuvant (*i.e.*, Proteosome) and liposaccharide.

In the preferred embodiment, the final liposaccharide content by weight as a percentage of the total Proteosome protein can be in a range from about 1% to about 500%, more preferably in range from about 10% to about 200%, or in a range from about 30% to about 150%. Another embodiment of the instant invention includes an adjuvant wherein the Proteosomes are prepared from *Neisseria meningitides* and the

liposaccharide is prepared from *Shigella flexneri* or *Plesiomonas shigelloides*, and the final liposaccharide content is between 50% to 150% of the total Proteosome protein by weight. In another embodiment, Proteosomes are prepared with endogenous lipooligosaccharide (LOS) content ranging from about 0.5% up to about 5% of total OMP. Another embodiment of the instant invention provides Proteosomes with endogenous liposaccharide in a range from about 12% to about 25%, and in a preferred embodiment between about 15% and about 20% of total OMP. The instant invention also provides a composition containing liposaccharide derived from any Gram-negative bacterial species, which may be from the same Gram-negative bacterial species that is the source of Proteosomes or is a different bacterial species.

In certain embodiments, the Proteosome or Protollin to antigen ratio in the mixture is greater than 1:1, greater than 2:1, greater than 3:1 or greater than 4:1. The ratio can be as high as 8:1 or higher. In other embodiments, the ratio of Proteosomes to antigen of the immunogenic composition ranges from about 1:1 to about 1:500, preferably the ratio is at least 1:5, at least 1:10, at least 1:20, at least 1:50, or at least 1:100. An unexpected result of the instant invention is that Protollin:antigen ratios could be reduced dramatically (from 1:2 to 1:200) with no significant effect on the ability of an antigen to elicit an immune response.

ANTIGENS

The invention relates to immunogenic compositions that contain one or more antigens, which can be used to elicit an immune response, such as a protective immune response. The invention further relates to methods for treating and preventing microbial infections by administering to a subject one or more antigens or fragments or variant thereof, fusion protein, multivalent immunogen, or a mixture of such immunogens at a dose sufficient to elicit an immune response (cellular or humoral) specific for the antigen (which may be a protective immune response), as described herein. The antigens are preferably from clinically relevant microorganisms, such as bacteria, viruses (*e.g.*, Influenza, Measles, Coronavirus), parasites (*e.g.*, Trypansome, Plasmodium, Leishmania, pathogenic bacteria) fungi (*e.g.*, Aspergillus, Candida,

Coccidioides, Cryptococcus), and the like. For example, the antigen may be from bacteria, such as the causative agent of anthrax (*Bacillus anthracis*), plague (*Yersinia pestis*), stomach cancer (*Helicobacter pylori*), Q fever (*Coxiella burnetii*), whooping cough (*Bordetella pertussis, e.g.*, FHA), botulism (*Clostridium botulinum*, types A, B,C, D, and E), gas gangrene (*Clostridium perfringens*), tularemia (*Francisella tularensis*), meliodiosis (*Pseudomonas pseudomallei*), brucellosis (*Brucella suis, B. abortus, B. canis, B. melitensis*), sexually transmitted diseases (*Chlamydia trachomatis* or *Neisseria gonorrhea*), toxin producing organisms, such as *Clostridium perfringens* (epsilon toxin, one of twelve protein toxins produced by these gram positive, anaerobic spore-forming bacteria).

There are five strains of *C. perfringens* (A through E), each producing a unique set of toxins; epsilon toxin is produced by the B and D types. Epsilon toxin is known as a performing polypeptide that causes potassium and fluid leakage from cells. In addition to epsilon toxin the A through E types may produce one or more of Alpha, beta, epsilon or iota toxin. The epsilon toxin can be disseminated by ingestion of contaminated food or water, or by inhalation of aerosolized toxin. Another exemplary toxin of concern is Staphylococcal enterotoxin B (SEB) produced by *Staphylococcus aureus*. SEB is known to cause illness if inhaled and maybe the cause of food poisoning. The immune system of SEB intoxicated individuals is compromised and although not frequently associated with death, there is significant and wide spread incapacitating illness, such as high fever, chills, headache, muscle aches, inflammation of the lining of the eyelids, nausea, vomiting and diarrhea. There is no vaccine or anti-SEB currently available.

In certain embodiments, isolated LPS may be an antigen, for example, LPS isolated from *P. gingivalis*, which may be formulated with Proteosomes for use in stimulating an immune response to *P. gingivalis* for treating or preventing gum disease, periodontal disease, tooth decay, and the like.

Other representative examples include antigens from certain viruses, such as Norwalk virus, small pox virus, West Nile virus, SARS virus, respiratory syncytial virus, and the like. Examples of Filoviruses include Marburg virus and Ebola

virus; examples of Flaviviruses include Dengue virus, Yellow Fever virus, Japanese encephalitis virus, Russian spring-summer encephalitis virus, St. Louis encephalitis virus; examples of Arenaviruses include Junin (Argentine hemorrhagic fever) virus; Lassa fever virus, lymphocytic choriomeningitis virus, Machupo (Bolivian hemorrhagic fever) virus; examples of Bunyaviruses include Crimean-Congo hemorrhagic fever virus, Hantaan (Korean hemorrhagic fever) virus, Rift Valley fever virus; examples of Picornavirus includes Hepatitis A virus, polio virus; examples of orthomyxovirus includes influenza virus A, B, C (*e.g.*, HA antigen), Thogoto virus; examples of alphaviruses include Chikungunya virus, Eastern encephalitis virus, Venezuelan encephalitis virus, Western encephalitis virus. Exemplary fungi include *Candida albicans, Aspergillus spp., Coccidioides immites, Histoplasma capsulatum, Norcardia farcinica*; and exemplary parasites include the causative agents of trypanosomiasis, leishmania, pneumonic plague, and lyme disease (*Borrellia burgdorferi*). In addition, antigens of interest may be toxic agents (*e.g.*, ricin, botulinum).

As described herein, the antigens can be prepared recombinantly, synthetically, isolated from a biological source, recombinantly or chemically modified, and any combination thereof. Microbial antigens or fragments thereof can be prepared from a variety of biological sources, such as tissues of an infected subject or cultured cell lines. Primary isolation may be from, for example, peripheral blood cells or from respiratory secretions. Preferably, the isolated microbes are amplified on primary cell cultures or on established cell lines known in the art. In certain embodiments, the antigens or fragments thereof are isolated from intact microbial particles. As used herein, the term "isolated" or "derived from" means that the material is removed from its original or natural environment. For example, a naturally occurring nucleic acid molecule or polypeptide present in a living animal or cell, or virus is not isolated, but the same nucleic acid molecule or polypeptide is isolated when separated from some or all of the co-existing materials in the natural system. For example, nucleic acid molecules could be contained in a vector and/or such nucleic acids could be part of a composition and still be isolated in that such a vector or composition is not part of the original nucleic acid molecule's natural environment. In other embodiments, peptides

or polypeptides, such as antigens or variants and fragments thereof, may be either partially purified or purified to homogeneity.

The present invention further provides methods for producing synthetic antigens, including fusion proteins. The immunogenic polypeptide components may be synthesized by standard chemical methods, including synthesis by automated procedure. In general, immunogenic polypeptides or peptides are synthesized based on the standard solid-phase Fmoc protection strategy with HATU as the coupling agent. The immunogenic peptide can be cleaved from the solid-phase resin with trifluoroacetic acid containing appropriate scavengers, which also deprotects side chain functional groups. Crude immunogenic peptide may be further purified using preparative reverse phase chromatography. Other purification methods, such as partition chromatography, gel filtration, gel electrophoresis, or ion-exchange chromatography may be used. Other synthesis techniques known in the art may be employed to produce similar immunogenic peptides, such as the tBoc protection strategy, use of different coupling reagents, and the like. In addition, any naturally or non-naturally occurring amino acid or derivative thereof may be used, including D- or L-amino acids and combinations thereof.

As described herein, the microbial antigens or fragments thereof of the invention may be recombinant, wherein a desired antigen is expressed from a polynucleotide that is operably linked to an expression control sequence (*e.g.*, promoter) in a nucleic acid expression construct. For example, host cells (such as baculovirus and mammalian cell lines) containing the immunogen-encoding nucleic acid expression constructs can be cultured to produce recombinant protein immunogens, or fragments thereof. The antigens may be further fused or conjugated to an amino acid sequence, which sequence may be a hydrophobic anchor or foot to facilitate or otherwise enhance non-covalent association with Projuvant or Protollin. The antigen polypeptides may comprise any portion of such polypeptides that have at least one epitope capable of eliciting a protective immune response (cellular or humoral) against microbial infection. Immunogenic polypeptides of the instant invention may also be arranged or combined in a linear form, and each immunogen may

or may not be reiterated, wherein the reiteration may occur once or multiple times. In addition, a plurality of different immunogenic polypeptides (*e.g.*, protein variants, or fragments thereof) can be selected and mixed or combined into a cocktail composition to provide a multivalent vaccine for use in eliciting a protective immune response.

To provide more detail about certain exemplary antigens for use in the immunogenic compositions of the instant disclosure, the following descriptions is provided.

A. PLAGUE

Plague, also known as the Black Death, is a disease with a long history. The plague pandemic of the Middle Ages killed up to 30 million people in Europe. Natural outbreaks still occur in endemic areas throughout the world, but because of the availability of antibiotic treatments, the disease causes little morbidity and death is a rare outcome. The fear surrounding plague, however, has been recently heightened with the possibility that it could be used as a biological warfare agent.

Bubonic plague are spread to humans through bites from fleas infected with *Yersinia pestis*, are relatively easy to diagnose and, therefore, treat effectively with anti-bacterials. The most fatal form of plague, pneumonic plague, is transmitted person-to-person by the respiratory route, or as the result of spread of bubonic or septicemic plague, by an infected and untreated person. Treatment of pneumonic plague after exposure is more problematic because of its rapid course - unless antibacterial treatment begins within 24 hours of showing symptoms of infection, most people who inhale *Y. pestis* will die. With the growing concern over the possible intentional transmission of plague, high priority has been given to the development of a safe vaccine capable of protecting against the most virulent pneumonic form of plague.

In some embodiments, the present invention provides a method for eliciting an immune response against plague, comprising administering to a subject in need thereof a therapeutically effective amount of an immunogenic composition, wherein said immunogenic composition comprises Proteosomes, liposaccharide and one or more plague antigens, or variants or fragments thereof. In other embodiments,

the present invention provides a method for treating or preventing a plague infection, comprising administering to a subject in need thereof a therapeutically effective amount of an immunogenic composition, wherein said immunogenic composition comprises Proteosomes, liposaccharide and one or more plague antigens or variants or fragments thereof, wherein the plague infection is treated or prevented. In certain embodiments, the plague antigen used in the immunogenic composition can comprise an F1 antigen or a V antigen from *Yersinia pestis*, or an F1-V antigen fusion protein antigen, or a combination thereof.

B. SMALL POX

In one embodiment, Protollin formulations comprising viral antigens that evoke a protective immune response against infection by, for example, variola virus causing smallpox disease in man is contemplated herein. Variola virus is the most notorious member of the virus family termed the Poxviridae and has historically had a significant and dramatic effect on human civilization. The Poxviridae is a large family of DNA viruses, which replicate in the cytoplasm of infected host cells. Infection with Variola virus kills 20-30% of those infected, and is highly contagious. Even though twenty years of prophylactic immunizations with cowpox and vaccinia virus vaccines have essentially eradicated occurrence of smallpox disease in man by the late 1970s there remains concern that smallpox virus could reemerge as a significant and deadly disease either from a naturally occurring virus population or as a consequence of intentional introduction into an un-expecting population as a biological warfare agent.

Virus particles purified from infected host cells are composed of as many as 30 or more discreet polypeptide bands when resolved by polyacrylamide gel electrophoresis. The genomic sequence of the Copenhagen strain of vaccinia virus contains about 185 unique, nonoverlapping open reading frames (ORFs) of more than 65 amino acids. By convention, the naming of the orthopoxvirus ORFs consists of using the HindII restriction endonuclease fragment letter followed by the ORF number (from left to right) and L or R depending on the direction of the ORF. The complete and nearly identical sequences of the India and Bangladesh strains of variola virus also

contain about 187 ORFs of which 150 have more than 90% sequence identity to those of vaccinia virus. One or more of he polypeptides encoded by vaccinia virus ORFs with 90% sequence identity to variola virus encoded polypeptides is assumed to mediate the immune response responsible for protection following vaccinia virus immunization. By way of example but not in limitation, a number of ORFs are known to encode extracellular virus-specific polypeptides that may be involved in eliciting a protective immune response. They include, the non-glycosylated 41.8 kilodalton protein encoded by the F13L ORF; the N-glycosylated 19.5 kilodalton protein encoded by the A34R ORF; the 25.1 kilodalton protein encoded by the A36R ORF; the N- and O-glycosylated hemagglutinin protein encoded by the A56R ORF; and the glycosylated 35.1 kilodalton protein encoded by the B5R ORF. The immune response to one or more of these proteins is expected to elicit a humoral or cell mediated immune response which may protect from variola virus infection or reduce the pathological effects of infection. An infected host immune response preferentially involving a balanced or fine tuned TH1/Th2 cytokine profile is expected to be effective in preventing or treating variola virus infection and occurrence of smallpox disease.

Four members of the Orthopoxvirus cause human infection, they are monkeypox, cowpox, variola and vaccinia viruses. Cowpox and vaccinia virus have been widely used to vaccinate humans against smallpox infection because they produced less serious complications compared to infection by variola virus. To a large extent, smallpox has been eradicated from being a serious health threat, and continued wide spread vaccination of the general population has been halted. However, reemergence of a natural virus or the rapid and wide deployment of a smallpox virus as a biological threat agent, and the possible unwanted side effects resulting from vaccination with live virus creates the need for the development of alternative vaccines against small pox.

To date, there is no non-living sub-unit vaccine against smallpox virus (i.e., variola virus) infection. Current vaccine formulations require the use of attenuated live virus to evoke a protective immune response, often with unwanted side effects. In certain embodiments, the present invention provides a method for eliciting an immune

response against small pox, comprising administering to a subject in need thereof a therapeutically effective amount of an immunogenic composition, wherein said immunogenic composition comprises Proteosomes, liposaccharide and one or more small pox antigens or a small pox viral particle extract. In other embodiments, the present invention provides a method for treating or preventing a small pox infection, comprising administering to a subject in need thereof a therapeutically effective amount of an immunogenic composition, wherein said immunogenic composition comprises Proteosomes, liposaccharide and one or more small pox antigens or a small pox viral particle extract, wherein the small pox infection is treated or prevented.

c. RICIN

In yet another aspect of the current invention, Proteosome-based vaccine compositions capable evoking an immune response to non-infectious, toxic or poisonous agents are contemplated herein. Such non-infectious agents include, for example, ricin toxin produced by the bean plant Ricinus communis, which is poisonous to humans, animals and insects. Ricin is a cytotoxin produced by castor beans, poisoning by inhalation or ingestion is caused by ricin. Ricin poisoning can, depending on dose, begin within a few hours of ingestion, causing abdominal pain, vomiting, diarrhea. Ricin poisoning, if unchecked, develops within several days into severe dehydration and a decrease in blood pressure resulting in death within 3-5 days after ingestion (or inhalation). Ricin is a potent toxin that is fairly easily produced and can cause poisoning (toxicity) by inhalation as a small particle or following ingestion. Ricin is also quite stable. Symptoms of ricin poisoning include weakness, fever, cough and pulmonary edema followed by severe respiratory distress and death from hypoxemia. In rodents, the histopathology of aerosol exposure is characterized by necrotizing airway lesions causing tracheitis, bronchitis and interstitial pneumonia with perivascular and aleveolar edema. In rodent, ricin is more toxic by inhalation than by other routes of exposure. Symptoms of ricin poisoning in a significant number of geographically localized individuals may suggest exposure to aerosolized ricin.

Cytotoxicity is mediated by the inhibition of protein synthesis through a mechanism that specifically and irreversibly inactivates the eukaryotic ribosome. Accordingly, ricin is also known a ribosome-inactivating protein (RIP). The RIPs such as ricin are generally made up of two disulfide bonded polypeptide chains. One polypeptide chain mediates binding to cells and uptake into the cytoplasm while the other chain functions to inhibit protein synthesis. In certain embodiments, the present invention provides a method for eliciting an immune response against ricin toxin, comprising administering to a subject in need thereof a therapeutically effective amount of an immunogenic composition, wherein said immunogenic composition comprises Proteosomes, liposaccharide and one or more ricin toxin antigens or fragments or variant thereof, wherein the ricin toxin antigen elicits a neutralizing antibody response. Thus, proteosome vaccine formulations comprising ricin immunogenic polypeptides that modulate the host Th1/Th2 immune response, and inactivates or attenuates toxin activity, are contemplated herein.

C. BOTULINUM

Botulism is caused by intoxication with any of the seven distinct neurotoxins produced by the bacillus, *Clostridium botulinum*. The botulinum toxins are proteins with molecular mass of approximately 150,000 KDa, which bind to the presynaptic membrane of neurons at peripheral cholinergic synapses to prevent release of acetylcholine and block neurotransmission. The blockade is most evident clinically in the cholinergic autonomic nervous system and at the neuromuscular junction. Symptoms of inhalation botulism may begin as early as 24-36 hours following exposure or as late as several days. Initial signs and symptoms include ptosis, generalized weakness, lassitude, and dizziness. Development of respiratory failure may be abrupt.

In certain embodiments, the present invention provides a method for eliciting an immune response against botulinum toxin, comprising administering to a subject in need thereof a therapeutically effective amount of an immunogenic composition, wherein said immunogenic composition comprises Proteosomes,

liposaccharide and one or more botulinum toxin antigens or fragments or variant thereof, wherein the botulinum toxin antigen elicits a neutralizing antibody response.

IMMUNOGENIC COMPOSITIONS AND METHODS OF USE

The invention also relates to immunogenic compositions that contain one or more antigens, which can be used to elicit an immune response, such as a protective immune response. The invention further relates to methods for treating and preventing microbial infections or effects of toxic agents by administering to a subject one or more antigens or fragments thereof, fusion protein, multivalent immunogen, or a mixture of such antigens at a dose sufficient to elicit an immune response (cellular or humoral) specific for the antigen(s) (which may be a protective immune response), as described herein. Antigens and variants thereof, or a cocktail of such immunogens are preferably part of a composition comprising an adjuvant, such as Projuvant or Protollin, when used in the methods of the present invention. In one embodiment, the immunogenic compositions of the instant invention may further comprise one or more additional microbial antigens, such as viral antigens, bacterial antigens, parasitic antigens, or a combination thereof. For example, an immunogenic composition for plague or anthrax may also include antigens for rubella and mumps antigens.

The immunogenic compositions may further include a pharmaceutically acceptable vehicle, carrier, diluent, or excipient, in addition to one or more antigen or fragment thereof and, optionally, other components. For example, pharmaceutically acceptable carriers or other components suitable for use with an immunogenic composition of this invention include a thickening agent, a buffering agent, a solvent, a humectant, a preservative, a chelating agent, an additional adjuvant, and the like, and combinations thereof.

In addition, the pharmaceutical composition of the instant invention may further include a diluent such as water or phosphate buffered saline (PBS). Preferably, diluent is PBS with a final phosphate concentration range from about 0.1 mM to about 1 M, more preferably from about 0.5 mM to about 500 mM, even more preferably from about 1 mM to about 50 mM, and most preferably from about 2.5 mM to about 10 mM;

and the final salt concentration ranges from about 100 mM to about 200 mM and most preferably from about 125 mM to about 175 mM. Preferably, the final PBS concentration is about 5 mM phosphate and about 150 mM salt (such as NaCl). In certain embodiments, any of the aforementioned immunogenic compositions comprising a cocktail of antigens and an adjuvant (such as Projuvant or Protollin) of the instant invention are preferably sterile.

The compositions can be sterilized either by preparing them under an aseptic environment or they can be terminally sterilized using methods available in the art. Many pharmaceuticals are manufactured to be sterile and this criterion is defined by the USP XXII <1211>. Sterilization in this embodiment may be accomplished by a number of means accepted in the industry and listed in the USP XXII <1211>, including gas sterilization, ionizing radiation or filtration. Sterilization may be maintained by what is termed aseptic processing, defined also in USP XXII <1211>. Acceptable gases used for gas sterilization include ethylene oxide. Acceptable radiation types used for ionizing radiation methods include gamma, for instance from a cobalt 60 source and electron beam. A typical dose of gamma radiation is 2.5 MRad. When appropriate, filtration may be accomplished using a filter with suitable pore size, for example 0.22 μm and of a suitable material, for instance Teflon®. The term "USP" refers to U.S. Pharmacopeia (*see* www.usp.org; Rockville, MD). Due to the fact that Proteosomes or OMP-LPS result in particles small enough that the immunogenic compositions of the invention can be filtered through a 0.8 μ filter, a 0.45 μ filter, or a 0.2 μ filter. Thus, in preferred embodiments the immunogenic compositions of this invention are sterilized by filtration. This is highly advantageous as it is desirable to eliminate any complications by virtue of the presence of such contaminants.

The present invention also pertains to methods for treating or preventing a microbial infection, comprising administering to a subject in need thereof an immunogenic composition comprising an adjuvant and one or more antigens, wherein the adjuvant comprises either Proteosomes or OMP-LPS. In another embodiment, the immunogenic compositions of this invention may be used to elicit an immune response (cellular or humoral or both, which may favor a Type 1 or Type 2 cellular response). A

subject suitable for treatment or for eliciting an immune response with an antigen formulation may be identified by well-established indicators of risk for developing a disease or well-established hallmarks of an existing disease. As used herein, it should be understood that the terms "treat" and "ameliorate" refer to the therapeutic administration of a desired composition or compound, in an amount or for a time sufficient to treat, inhibit, attenuate, ameliorate, reduce, prevent or alter at least one aspect or marker of a disease, in a statistically significant manner. Infections that may be treated, ameliorated or prevented with an antigen of the subject invention include those caused by or due to any of the microbes described herein, whether the infection is primary, secondary, opportunistic, or the like. Other exemplary antigens are the toxins or variants thereof described herein.

As described herein, the antigens can be prepared recombinantly, synthetically, isolated from a biological source, recombinantly or chemically modified, and any combination thereof. Microbial antigens or fragments thereof can be prepared from a variety of biological sources, such as tissues of an infected subject or cultured cell lines. Primary isolation may be from, for example, peripheral blood cells or from respiratory secretions. Preferably, the isolated microbes are amplified on primary cell cultures or on established cell lines known in the art. In certain embodiments, the antigens or fragments thereof are isolated from intact microbial particles. As used herein, the term "isolated" or "derived from" means that the material is removed from its original or natural environment. For example, a naturally occurring nucleic acid molecule or polypeptide present in a living animal or cell, or virus is not isolated, but the same nucleic acid molecule or polypeptide is isolated when separated from some or all of the co-existing materials in the natural system. For example, nucleic acid molecules could be contained in a vector or such nucleic acids could be part of a composition and still be isolated in that such a vector or composition is not part of the original nucleic acid molecule's natural environment. In other embodiments, peptides or polypeptides, such as antigens or variants and fragments thereof, may be either partially purified or purified to homogeneity.

The present invention further provides methods for producing synthetic antigens, including fusion proteins. The immunogenic polypeptide components may be synthesized by standard chemical methods, including synthesis by automated procedure. In general, immunogenic polypeptides or peptides are synthesized based on the standard solid-phase Fmoc protection strategy with HATU as the coupling agent. The immunogenic peptide can be cleaved from the solid-phase resin with trifluoroacetic acid containing appropriate scavengers, which also deprotects side chain functional groups. Crude immunogenic peptide may be further purified using preparative reverse phase chromatography. Other purification methods, such as partition chromatography, gel filtration, gel electrophoresis, or ion-exchange chromatography may be used. Other synthesis techniques known in the art may be employed to produce similar immunogenic peptides, such as the tBoc protection strategy, use of different coupling reagents, and the like. In addition, any naturally or non-naturally occurring amino acid or derivative thereof may be used, including D- or L-amino acids and combinations thereof.

As described herein, the microbial antigens or fragments thereof of the invention may be recombinant, wherein a desired antigen is expressed from a polynucleotide that is operably linked to an expression control sequence (*e.g.*, promoter) in a nucleic acid expression construct. For example, host cells (such as baculovirus and mammalian cell lines) containing the immunogen-encoding nucleic acid expression constructs can be cultured to produce recombinant protein immunogens, or fragments thereof. The antigens may be further fused or conjugated to an additional amino acid sequence, which may be a hydrophobic anchor or foot to facilitate or otherwise enhance non-covalent association of the antigen fusion with Projuvant or Protollin. The antigen polypeptides may comprise any portion of such polypeptides that have at least one epitope capable of eliciting a protective immune response (cellular or humoral) against a microbial infection. Immunogenic polypeptides of the instant invention may also be arranged or combined in a linear form, and each immunogen may or may not be reiterated, wherein the reiteration may occur once or multiple times. In addition, a plurality of different immunogenic

polypeptides (*e.g.*, protein variants, or fragments thereof) can be selected and mixed or combined into a cocktail composition to provide a multivalent vaccine for use in eliciting a protective immune response.

Methods for preparing the immunostimulatory compositions, immunomodulatory compositions and immunogenic compositions of the instant disclosure are described herein and are known in art (*see, e.g.*, U.S. Patent Application Publications Nos. 2001/0053368 and 2003/0044425). The antigen(s) and adjuvant are formulated at specific initial ratios to optimize interaction (or cooperation) between the components resulting in non-covalent association (or non-specific juxtaposition) of a significant portion of the two components with each other. For example, a mixture of at least one antigen with a Proteosome (projuvant) or Protollin is prepared in the presence of detergent, and reducing or removal of the detergent from the mixture by diafiltration/ultrafiltration leads to association (or coordination) of the antigens with the adjuvant. In certain embodiments, the Protollin (or Proteosome) to antigen ratio in the mixture ranges from about 1:1 to about 5:1. The ratio can be as high as 8:1 or higher. In certain other embodiments, the Protollin (or Proteosome) to antigen ratio in the mixture ranges from about 1:1 to about 1:500, or in a range of about 1:2 to about 1:200, or in a range of about 1:2 to about 1:100, or in a range of about 1:5 to about 1:50, or in a range of about 1:2 to about 1:20, or at least 1:5, 1:10, 1:20 or 1:100. The detergent-based solutions of the two components may contain the same detergent or different detergents, and more than one detergent may be present in the mixture subjected to ultrafiltration/diafiltration. Suitable detergents include Triton®, Empigen® BB, and Mega-10. Other detergents can also be used (*e.g.*, octoglucoside). The detergents serve to solubilize the components used to prepare the composition. The use of a mixture of detergents may be particularly advantageous. This mixture is, of course, removed or the concentration is reduced by diafiltration/ultrafiltration prior to final formulation.

The immunogenic compositions that contain one or more antigens and a Proteosome-based adjuvant of the invention may be in any form that allows for the composition to be administered to a subject, such as a human or animal. For example, immunogenic compositions of the present invention may be prepared and administered

as a liquid solution or prepared as a solid form (*e.g.*, lyophilized), which may be administered in solid form, or resuspended in a solution in conjunction with administration. The immunogenic polypeptide compositions are formulated to allow the active ingredients contained therein to be bioavailable upon administration of the composition to a subject or patient or bioavailable via slow release. Compositions that will be administered to a subject or patient take the form of one or more dosage units, where for example, a drop may be a single dosage unit, and a container of one or more compounds of the invention in aerosol form may hold a plurality of dosage units. In certain preferred embodiments, any of the aforementioned pharmaceutical compositions comprising an immunogen or cocktail of immunogens (*i.e.*, anitgens) of the invention are in a container, preferably in a sterile container. The design of a particular protocol for administration, including dosage levels and timing of dosing are determined by optimizing such procedures using routine methods well known to those having ordinary skill in the art.

In one embodiment, the immunogenic composition is administered nasally. Other typical routes of administration include enteral, parenteral, transdermal/transmucosal, nasal, and inhalation. The term "enteral", as used herein, is a route of administration in which the immunogenic composition is absorbed through the gastrointestinal tract or oral mucosa, including oral, rectal, and sublingual. The term "parenteral", as used herein, describes administration routes that bypass the gastrointestinal tract, including intraarterial, intradermal, intramuscular, intranasal, intraocular, intraperitoneal, intravenous, subcutaneous, submucosal, and intravaginal injection or infusion techniques. The term "transdermal/transmucosal", as used herein, is a route of administration in which the immunogenic composition is administered through or by way of the skin, including topical. The terms "nasal" and "inhalation" encompass techniques of administration in which an immunogenic composition is introduced into the pulmonary tree, including intrapulmonary or transpulmonary. Preferably, the compositions of the present invention are administered nasally.

For example, as described herein, intranasally administered F1-V combined with Protollin elicited comparable serum anti-F1-V IgG titers to those

induced by intramuscular injection of F1-V formulated with Alhydrogel®. However, unlike Alhydrogel®/F1-V, Protollin/F1-V also elicited specific IgA and IgG responses as detected in lung lavage fluids. Immunized mice were subsequently challenged with aerosolized *Y. pestis*. Protollin/F1-V immunized mice were 80% protected against high dose (255 LD_{50}) challenge as compared to 60% in the Alhydrogel/F1-V group, while both vaccines were 90-100% protective against challenge with a lower dose (170 LD_{50}) of the organism.

All of the U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification and/or listed in the Application Data Sheet, are incorporated herein by reference, in their entirety. The invention having been described, the following examples are intended to illustrate, and not limit, the invention.

EXAMPLES

EXAMPLE 1

ELISA ASSAYS TO EVALUATE SERUM AND MUCOSAL IMMUNE RESPONSE

ELISAs were used to determine total and antigen-specific IgG, IgA and IgM titers in biosamples obtained from animals immunized with test immunostimulatory or immunogenic formulations according to the instant application, and controls. Samples include serum, nasal and lung mucosal washes in mice and rabbits. A standard ELISA protocol was used to determine linearity, specificity, sensitivity and reproducibility. Briefly, serial dilutions of the test samples (serum and lavage fluids) were added to the wells of ELISA plates coated with purified antigen, or derivatives thereof. Antigen-specific antibodies that adhere to the immobilized antigen were detected with animal and antibody subtype specific horse radish peroxidase (HRP) conjugated antibodies. Following HRP antibody interaction and washing, the amount of bound HRP antibody was detected following incubation with a HRP substrate (TMB) and measuring absorbance at 490 nm. Antibody concentrations in the test samples were calculated from standard curves, run in parallel, using purified standard antibodies for IgA, IgM, IgG, IgG1 and IgG2a. Where appropriate, specific antibody levels in mucosal wash fluid samples are standardized and normalized by expressing the specific antibodies detected in comparison to the total amount of IgA or IgG in the sample as measured in a separate assay. This technique has resulted in superior reproducibility and consistency in assays performed on nasal wash fluids collected in human clinical trials. ELISA data is expressed as geometric means at 95% confidence levels with statistical analysis using log-transformed data.

EXAMPLE 2

ANALYSIS OF MUCOSAL ANTIBODY PRODUCTION

Lung and nasal washes in mice and rabbits were collected to analyze the immune response to immunostimulatory or immunogenic formulations according to the

instant application. In mice, nasal washes and lung lavage were performed by cannulating the trachea and 1 ml PBS supplemented with 0.1% bovine serum albumin and protease inhibitors (General Use Protease Inhibitor Cocktail; Sigma Chemicals containing 0.2 mM AEBSR, 1μg/mL aprotinin, 3.25 μM bestatin, 10 μM Leupeptin) were pumped upwards through the trachea. Fluid emerging from the nostrils was collected, vortexed and then centrifuged to remove tissue and cell debris. The supernatants were stored at -70°C until assayed. A cannula reinserted into the trachea and keeping the cannula directed toward the lung allowed the collection of lung fluids. Lungs were lavaged twice with 1.0 mL protease supplemented PBS, the fluid was collected and vortexed, and the cell debris removed by centrifugation. Lung and nasal washes were stored at -70°C until being assayed. Rabbit mucosal fluids were similarly collected, adjusting the volumes as appropriate. In certain experiments, after collecting mucosal samples, cervical and mediastinal lymph nodes were surgically removed and mononuclear cells isolated and cultured for ELISPOT antibody, cytokine and CMI assays as described herein.

EXAMPLE 3

IMMUNIZATION WITH PROTOLLIN FORMULATED WITH PLAGUE ANTIGEN F1-V

Examined is the ability of Proteosome:LPS (Protollin) compositions formulated with, for example, plague antigen (F1-V) to evoke an immune response capable of protecting against a lethal challenge with *Yersinia pestis*. The F1-V immune response was assessed by immunizing groups of twenty 6-8 week old female Swiss-Webster mice (Charles River, St-Constant, Quebec) on days 0 and 21. For immunization, freshly thawed aliquots of Protollin and F1-V solutions were mixed <16 hrs prior to immunization. For intranasal (i.n.) administration, mice were first lightly anesthetized by isoflurane inhalation, then 25 μl of vaccine or appropriate control samples (Protollin or F1-V alone) was applied to the nares (12.5 μl per nostril) of each mouse. In parallel experiments, mice were immunized intramuscular (i.m.) with 25 μl of F1-V adsorbed to 500mg of Alhydrogel® injected into hind limbs. Control i.m. injections were also performed. Thirty-five and 55 days thereafter, 10 mice from each

group were euthanized by asphyxiation with CO_2 and exsanguination. Serum was obtained and stored at -80°C until assay. Nasal wash and lung lavage samples were obtained and stored at -80°C until assay. Spleens were processed for *in vitro* restimulation and assessment of released cytokines. The remaining 10 mice from each group were challenged on day 35 or 55 by inhalation of 170-250 LD50 of aerosolized *Y. pestis* (Colorado 92 strain) to assess protection. Mice were monitored for 28 days after challenge for determination of morbidity and mortality.

Antibodies present in serum and lung lavage fluid samples obtained from mice immunized intranasally with two doses of F1-V antigen formulated with Protollin were compared with those from mice immunized i.n. with F1-V alone or with mice immunized intramuscularly with Alhydrogel®-adsorbed F1-V. The results are shown in Figures 1 and 2. Surprisingly, at all concentrations tested, all combinations of Protollin and F1-V were highly immunogenic and elicited F1-V specific serum IgG titers of between 1 mg/ml and 9 mg/ml (Figures 1 and 2). There were no significant differences in the specific IgG titers elicited by any combination of F1-V and Protollin concentrations or those elicited by intramuscular injection of 20μg of F1-V adsorbed onto Alhydrogel ($P > 0.05$). All specific serum IgG titers elicited by F1-V formulated vaccines were significantly higher than those elicited by i.n. administration of non-adjuvanted F1-V controls ($P \leq 0.001$), and no F1-V specific antibodies were detected in serum from control mice. An unexpected result of these experiments is that Protollin:antigen ratios could be reduced dramatically (from 1:2 to 1:200) with no significant effect on the ability of an antigen to elicit an immune response. Prior to this disclosure, one would have expected that reducing the amount of adjuvant would have resulted in a reduction in the immune response.

Lung lavage samples were assayed by ELISA to determine the level of specific anti-F1-V, anti-F1 and anti-V antibodies present (Figure 3). The results confirm that immunization by mucosal (*e.g.*, intranasal) routes is an efficient means of eliciting mucosal antibodies as all groups of mice immunized i.n. with F1-V antigen plus Protollin responded with high titers of F1-V specific lung IgA. In these experiments, the IgA responses were highest in lung lavage samples from mice

immunized with the highest dose of Protollin; ANOVA analysis indicated, again unexpected, that there were no significant differences in the IgA titers elicited by any combination of F1-V with Protollin. Non-adjuvanted F1-V, administered i.n., elicited IgA levels that were barely detectable; no secretory IgA was detected in samples from mice injected i.m. with Alhydrogel®-adsorbed F1-V. Assays of lung lavage samples against the F1 and V portions of the F1-V antigen indicated that the immune response was primarily directed against the V component of the F1-V fusion protein (Table 1). However, lung lavage samples also contained significant titers of F1-V specific IgG, even though the titers represented only a small percentage of the serum titers (range 0.11% – 0.56%; median 0.175%).

To determine if serum antibodies recognizing both the F1 and V components were elicited, sera from all mice immunized with the 20 μg dose of F1-V antigen were analyzed separately against F1 and V. In all instances, and at both sampling times, serum IgG and lung lavage fluid IgA antibodies specific for both the F1 and V portions of the F1-V antigen were detected (Table 1). There were no significant differences in the serum anti-F1 and anti-V antibody ratios, irrespective of the formulation or route of delivery of the F1-V antigen evaluated in these experiments.

Table 1. Ratios of anti-F1 to anti-V Antibodies in Serum and Lung Lavage fluids of Mice Immunized with Several Formulations of Plague Antigen F1-V

	F1-V+ 2.5μg Protollin	F1-V+ 1μg Protollin	F1-V + 0.25μg Protollin	F1-V i.n.	F1-V + Alhydrogel i.m.
Serum IgG d35	0.31	0.30	0.34	0.31	0.65
Serum IgG d55	0.25	0.26	0.33	0.58	0.29
Lung IgA d35	0.42	0.39	0.33	N/A	N/A
Lung IgA d55	0.49	0.29	0.32	N/A	N/A
Lung IgG d35	0.40	0.53	0.44	N/A	1.02
Lung IgG d55	0.48	0.48	0.46	N/A	1.03

EXAMPLE 4

CHALLENGE OF IMMUNIZED MICE WITH AEROSOLIZED *Y. PESTIS*

These experiments were designed to assess the level of immune protection elicited by intranasal immunization with F1-V formulated with Protollin. Mice were challenged by whole-body exposure to live aerosolized *Y. pestis*, and the level of protection from challenge was compared with that elicited by injection of F1-V adsorbed onto Alhydrogel, as well as control mice administered F1-V alone or Protollin alone, intranasally. In these experiments, only the results for mice immunized with the 20 μg doses of F1-V are shown in Figures 4 and 5. On day 35 and at a challenge dose of 170 LD50 *Y. pestis*, mice immunized intranasally with 5, 20 or 50 μg F1-V plus 1 or 2.5 μg Protollin were 100% protected against death, as were the mice injected with F1-V adsorbed onto Alhydrogel. Mice immunized i.n. with 5, 20 or 50 μg F1-V and 0.25 μg Protollin were 90%, 100% and 90% protected, respectively, while mice immunized i.n. with the same doses of F1-V without Protollin were only 30%, 40% and 40% protected, respectively. None of the control mice, which received Protollin alone, survived longer than 4 days post challenge. Survival for all mouse groups immunized with formulated F1-V was highly significant compared to survival in control mice or mice immunized with unformulated F1-V ($P \leq 0.05$ or better by Fisher's Exact Probability Test). Survival following challenge on day 55 was very similar to the day 35 data. All mice immunized with 2.5 μg of Protollin formulated with F1-V were completely protected against challenge, as were mice immunized by injection of F1-V adsorbed onto Alhydrogel®. Mice immunized with 1 μg of Protollin formulated with 50 μg or 20 μg of F1-V were also 100% protected, while all other combinations of Protollin and F1-V elicited 90% protection. In all mice immunized with formulated F1-V, the observed protection was highly significant ($P \leq 0.01$ or better) compared to mice immunized with non-adjuvanted F1-V (10-30% protection) or the control group of mice in which there were no survivors. Mice immunized i.n. with 50 μg of F1-V with or without 1μg of Protollin, or injected with 20 μg of F1-V adsorbed onto Alhydrogel®, were challenged on day 55 by whole body exposure to 255 LD50 aerosolized live *Y. pestis*. 80%, 20% and 60% respectively of the mice survived the high dose lethal

challenge while control mice given Protollin alone all died. Immunization with formulated F1-V induced significant protection against death compared to control mice ($P \leq 0.001$ for nasal F1-V plus Protollin; $P \leq 0.01$ for injected F1-V). Nasal immunization with F1-V plus Protollin was also significantly better at protecting mice against death than immunization with F1-V alone ($P \leq 0.05$), but this was not the case for injected F1-V when the induced protection was not significantly better than that induced by i.n. F1-V without Protollin ($P = 0.095$).

EXAMPLE 5

DETERMINATION OF CYTOKINE PROFILE AFTER IMMUNIZATION WITH PROTOLLIN:PALGUE ANTIGEN

To compare the phenotype (type 1 or type 2) of the adaptive immune response elicited by i.n. adminstered Protollin or injected Alhydrogel® adjuvanted F1-V vaccine, splenocytes from selected groups of immunized mice were re-stimulated in vitro with F1-V and the amount of IFN-γ, TNF-α and IL-5 cytokines released into culture supernatants were measured (Figure 6). Splenocytes from mice immunized i.n. with F1-V (50 μg) mixed with Protollin (1 μg) responded to *in vitro* re-stimulation by secreting high levels of both IFN-γ and TNF-α, and a very low amount of IL-5 was also detected. In contrast, splenocytes from mice immunized by injection of F1-V (20 μg) adsorbed onto Alhydrogel responded by secreting comparatively lower amounts of IFN-γ and TNF-α, although a significant amount of IL-5 was detected. Thus, the cytokine profile elicited by i.n. administration of Protollin formulated (adjuvanted) with F1-V antigen was consistent with eliciting a type 1 immune response, whereas the cytokine profile induced by i.m. injection of F1-V antigen formulated with Alhydrogel® is more consistent with a type 2 response.

EXAMPLE 6

PROTOLLIN:ANTHRAX IMMUNOGENIC FORMULATIONS EVOKE A NEUTRALIZING IMMUNE RESPONSE

Protollin formulations with a recombinant Protective Antigen (rPA) of *Bacillus anthracis* were evaluated for the ability to induce an immune response capable eliciting a statistically significant reduction in PA-mediated macrophage killing, using a cell culture assay system. Mice immunized i.n. with 5 μg or 25 μg rPA (List Laboratories) admixed with 1 μg of Protollin (on days 0, 14) showed specific anti-rPA serum IgG and lung IgA levels significantly higher than those of mice that were i.n. immunized with 5 μg or 25 μg of rPA alone ($p<0.05$) (Figure 7), indicating that adjuvanticity of low levels of Protollin is also effective with rPA antigen. Mucosal IgA levels in the Protollin alone and rPA alone control groups were below the detection level of this assay.

The ability of specific anti-rPA antibodies to neutralize PA-mediated macrophage killing was evaluated. An anthrax PA neutralization assay was also performed as described using serum and lung lavage fluid samples from these animals. For this assay, 2 x 105 RAW264.7 cells were plated out in sterile 96 well plates and incubated at 37°C for 24 h in 5% CO2. Serial dilutions of serum or lung lavage fluid samples (from PA immunized animals) were incubated with a PA solution for 1 h at 37°C, which was then added to the wells containing cells. Thereafter, a solution of LF was added to the wells and the plates incubated at 37°C in 5% CO_2 for 4 h. A solution of MTT (to measure cell viability) was then added to each well and the plates incubated for a further 4h at 37°C in 5% CO_2. The reaction was stopped by addition of 20% SDS in 50% DMF pH 4.3 and the plates read at 570 nm with a reference at 690 nm. This assay showed that the Proteosome-adjuvanted i.n. vaccine of rPA with Protollin elicited comparable levels of antibodies that neutralized rPA activity as did the i.m. alum-adjuvanted vaccine (Figure 8). However, a notable distinction between samples is the presence of neutralizing IgA antibodies present in lung lavage samples from mice immunized with the Protollin formulation rPA.

EXAMPLE 7

PREPARATION OF ADDITIONAL ANTHRAX VACCINE FORMULATIONS

Nasal Protollin anthrax vaccines were made by admixing the anthrax PA antigens with soluble pre-formed Proteosome plus LPS (*i.e.*, Protollin) prior to immunization. Both rPA and rPA-anchor antigens are evaluated with several versions of Protollin to determine the formulation(s) having the optimal Protollin to immunogenic antigen ratio. Control formulations include, for example, Protollin alone or mixed with one or more control antigens (as appropriate), such as recombinant streptococcal protein that contains or lacks a hydrophobic anchor sequence. Accordingly, formulations of Protollin containing LPS from different sources, various Proteosome:LPS ratios, and various Protollin:rPA antigen ratios are evaluated. rPA-anchor are formulated with Proteosomes with very low levels of LPS (<2% by weight). In certain embodiments, the Proteosome adjuvant preparations do not have exogenous LPS added. Previosuly, Proteosomes themselves have been used extensively in pre-clinical toxicity studies as well as in close to 500 persons in Phase 1 and Phase 2 safety, immunogenicity and experimental challenge trials of a Proteosome nasal influenza vaccine.

Process development of the preferred version of LPS (in terms of LPS bacterial source) and the preferred formulation of Protollin (in terms of LPS source and Proteosome:LPS ratio) are performed based on the results of the immunogenicity studies designed to identify the components of Protollin in terms of LPS source and Proteosome:LPS ratio. Fermentation of the preferred bacterial source for LPS is followed by LPS purification and analysis. The selected purified LPS is then mixed with Proteosome OMP particles at the selected ratio to form a preferred Protollin. The extent of association of the LPS with the OMPs is verified in specially developed "free-vs.-bound" assays using capillary electrophoresis, LPS "spiking" studies and other analyses, as necessary. Characterization of Protollin typically includes analyses of LPS content using KDO, NMR, and silver stain PAGE, for Proteosome OMP content using LC-MS, RP-HPLC, SDS-Page (C. Blue & Western Blot w/MAb & PAbs), N-terminal Sequencing, Amino Acid Analysis, total protein by Lowry or BCA, and MALDI-

TOFMS where appropriate, and for residual LPS, Nucleic Acids, and detergents using KDO, nucleic acid and HPLC assays, respectively.

EXAMPLE 8

CELL MEDIATED IMMUNITY ASSAY TO EVALUATE IMMUNIZATION AGAINST ANTHRAX

Cellular immune responses induced following immunization with the Proteosome-based PA vaccines is studied using a variety of methods. For example, T cell-derived cytokines are assessed on PA-re-stimulated purified or enriched T cells isolated from mouse spleen and/or mediastinal lymph nodes. Type 1 (*e.g.*, IFN-γ) and type 2 (*e.g.*, IL-4 and IL-5) cytokines are determined by one or more methods including ELISA, ELISPOT and intracellular cytokines by flow-cytometry. Proliferation of PA re-stimulated PBMC T cells from PBMC are used to evaluate CMI responses in rabbits due to lack of reagents specific for rabbit cytokines. T lymphocyte proliferation assays are used to measure the effect of immunization on clonal expansion and presence of memory lymphocytes in various animal models. Following animal sacrifice, mediastinal and cervical lymph nodes are removed surgically using standard techniques, the lymphocytes isolated and cultured and PA added to the isolated cells. Proliferation is measured by uptake of H^3-thymidine. Cells from animals immunized with sham vaccine are used as negative controls. Assay results are used to determine the effect of immunization on T cell differentiation in the lymph nodes involved in mucosal immunity, and correlated with efficacy of immunization as determined in anthrax challenge studies.

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

CLAIMS

1. A method for eliciting an immune response to an antigen, comprising administering to a subject in need thereof a therapeutically effective amount of an immunogenic composition, wherein said immunogenic composition comprises Proteosomes, liposaccharide and an antigen, wherein said antigen is from *Bacillus anthracis, Chlamydia trachomatis, Staphylococcus aureus, Clostridium perfringens,* or *Yersinia pestis*, such that an immune response to said antigen is elicited.

2. A method for treating or preventing a microbial infection, comprising administering to a subject in need thereof a therapeutically effective amount of an immunogenic composition, wherein said immunogenic composition comprises Proteosomes, liposaccharide and an antigen, wherein said antigen is from *Bacillus anthracis, Chlamydia trachomatis, Staphylococcus aureus, Clostridium perfringens,* or *Yersinia pestis*, such that a microbial infection is treated or prevented.

4. The method according to claim 1 or 2 wherein the antigen is recombinant.

5. The method according to claim 1 or 2 wherein the antigen is a Protective Antigen from *Bacillus anthracis.*

6. The method according to claim 1 or 2 wherein the antigen is an F1 antigen or a V antigen from *Yersinia pestis*, or a combination thereof.

7. The method according to claim 6 wherein the F1 antigen and V antigen are an F1-V antigen fusion protein.

8. The method according to claim 1 or 2 wherein the immunogenic composition comprises a plurality of different antigens.

9. The method according to claim 8 wherein the plurality of antigens comprise viral, bacterial, parasitic, toxin, or a combination thereof.

10. The method according to claim 1 or 2 wherein the immunogenic composition is administered by a route selected from the group consisting of mucosal, enteral, parenteral, transdermal, transmucosal, nasal, and inhalation.

11. The method according to claim 10 wherein the immunogenic composition is administered nasally.

12. The method according to claim 1 or 2 wherein the liposaccharide final content by weight as a percentage of Proteosome protein ranges from about 1% to about 500%.

13. The method according to claim 1 or 2 wherein the Proteosomes and liposaccharide are obtained from the same bacteria.

14. The method according to claim 1 or 2 wherein the Proteosomes and liposaccharide are from different bacteria.

15. The method according to claim 1 or 2 wherein the liposaccharide is from Gram-negative bacteria.

16. The method according to claim 15 wherein the liposaccharide is from Gram-negative bacteria selected from the group consisting of *Shigella* species, *Chlamydia* species, *Yersinia* species, *Pseudomonas* species, *Plesiomonas* species, *Escherichia* species, *Salmonella* species, or a combination thereof.

17. The method according to claim 1 or 2 wherein the Proteosomes are from *Neisseria* species.

18. The method according to claim 1 or 2 wherein the Proteosomes are from *Neisseria meningitides*, and the liposaccharide is from *Shigella flexneri*.

19. The method according to claim 1 or 2 wherein the ratio of Proteosomes to antigen of the immunogenic composition ranges from about 5:1 to about 1:500.

20. The method according to claim 19 wherein the ratio of Proteosomes to antigen of the immunogenic composition is at least 1:2.

21. The method according to claim 19 wherein the ratio of Proteosomes to antigen of the immunogenic composition is at least 1:10.

22. The method according to claim 1 wherein the immunogenic composition elicits a protective immune response.

23. The method according to claim 1 or 2 wherein the immunogenic composition further comprises a pharmaceutically acceptable carrier, excipient or diluent.

24. A composition comprising Proteosome, liposaccharide and an antigen, wherein the antigen is an F1-V fusion protein.

25. A composition comprising Proteosome, liposaccharide and an antigen, wherein the antigen is a Protective Antigen from *Bacillus anthracis*.

26. The composition according to claim 24 or 25 wherein the composition further comprises a pharmaceutically acceptable carrier, excipient or diluent.

27. The composition according to claim 24 or 25 wherein the liposaccharide final content by weight as a percentage of Proteosome protein ranges from about 1% to about 500%.

28. The composition according to claim 24 or 25 wherein the Proteosomes and liposaccharide are obtained from the same bacteria.

29. The composition according to claim 24 or 25 wherein the Proteosomes and liposaccharide are from different bacteria.

30. The composition according to claim 24 or 25 wherein the Proteosomes are from *Neisseria meningitides*, and the liposaccharide is from *Shigella flexneri*.

31. The composition according to claim 24 or 25 wherein the ratio of Proteosomes to antigen of the immunogenic composition ranges from about 5:1 to about 1:500.

32. The composition according to claim 31 wherein the ratio of Proteosomes to antigen of the immunogenic composition is at least 1:2.

33. The composition according to claim 31 wherein the ratio of Proteosomes to antigen of the immunogenic composition is at least 1:10.

34. A method for eliciting an immune response to an antigen, comprising administering to a subject in need thereof a therapeutically effective amount of an immunogenic composition, wherein said immunogenic composition comprises Proteosomes, liposaccharide and an antigen, wherein said antigen is from Poxvirus, Alphavirus, Flavivirus, Arenavirus, Bunyavirus, Filovirus, or Picornavirus, such that an immune response to said antigen is elicited.

35. A method for treating or preventing a microbial infection, comprising administering to a subject in need thereof a therapeutically effective amount of an immunogenic composition, wherein said immunogenic composition comprises Proteosomes, liposaccharide and an antigen, wherein said antigen is from Poxvirus, Alphavirus, Flavivirus, Arenavirus, Bunyavirus, Filovirus, or Picornavirus, such that a microbial infection is treated or prevented.

36. The method according to claim 34 or 35 wherein the antigen is from a Variola virus.

37. The method according to claim 34 or 35 wherein the antigen is from a Vaccinia virus.

38. A method for eliciting an immune response to an antigen, comprising administering to a subject in need thereof a therapeutically effective amount of an immunogenic composition, wherein said immunogenic composition comprises Proteosomes, liposaccharide and an antigen, wherein said antigen is a toxin, such that an immune response to said toxin is elicited.

39. The method according to claim 38 wherein the antigen is a botulinum toxin or fragment thereof.

40. The method according to claim 38 wherein the antigen is a ricin toxin or fragment thereof.

41. The method according to claim 38 wherein the antigen is a Staphylococcus enterotoxin B or fragment thereof.

42. A method for eliciting an immune response to an antigen, comprising administering to a subject in need thereof a therapeutically effective amount

of an immunogenic composition, wherein said immunogenic composition comprises an adjuvant and an antigen, wherein said adjuvant comprises Proteosomes and liposaccharide and said adjuvant to antigen ratio ranges from about 1:5 to about 1:500, such that an immune response to said antigen is elicited.

43. A method for treating or preventing a microbial infection, comprising administering to a subject in need thereof a therapeutically effective amount of an immunogenic composition, wherein said immunogenic composition comprises an adjuvant and an antigen, wherein said adjuvant comprises Proteosomes and liposaccharide and said adjuvant to antigen ratio ranges from about 1:5 to about 1:500, such that a microbial infection is treated or prevented.

44. The method according to claim 42 or 43 wherein the antigen is at least one of a bacterial, viral, or toxin antigen.

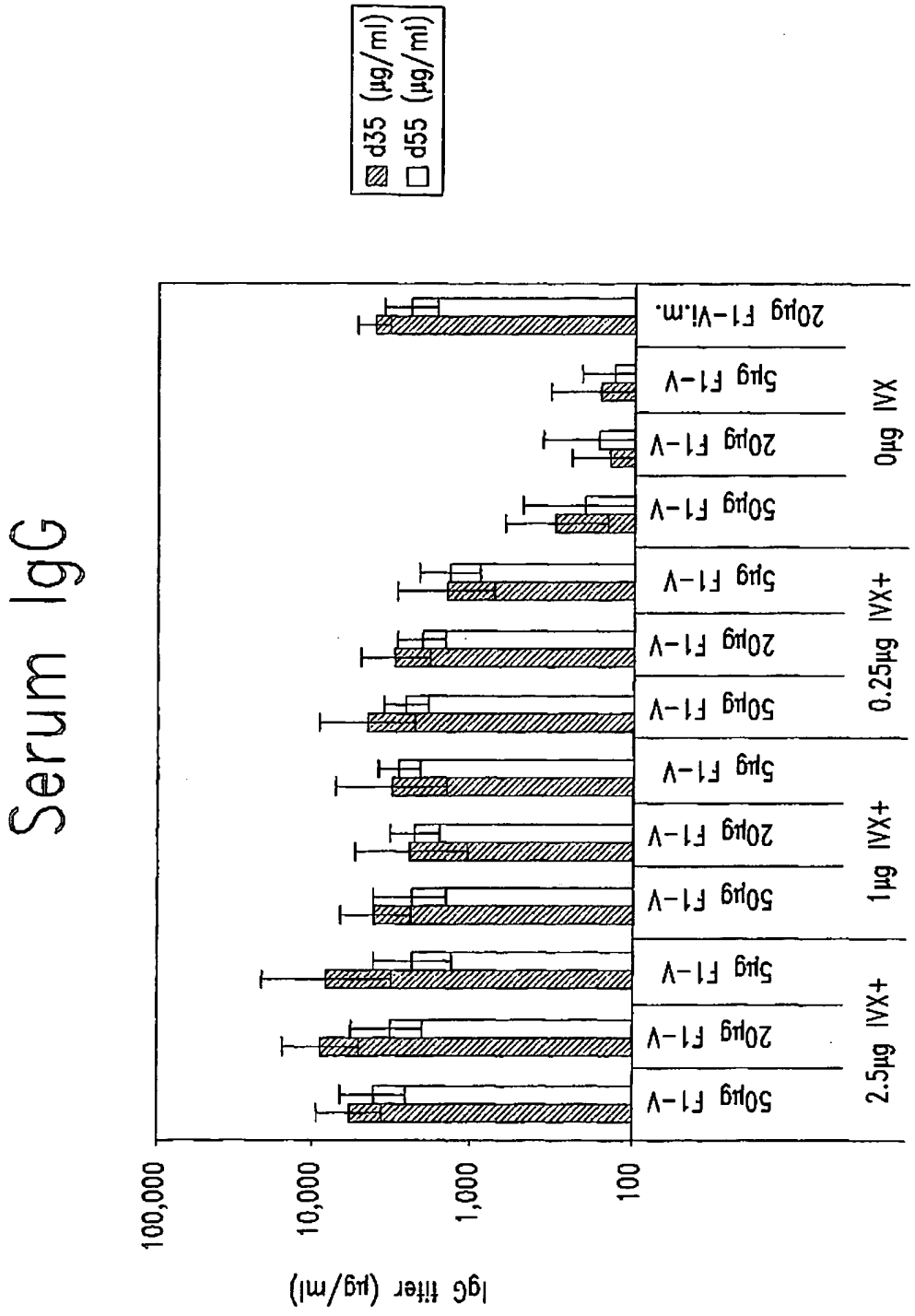
Serum IgG
d35 (µg/ml)
d55 (µg/ml)
IgG titer (µg/ml)
100,000
10,000
1,000
100
2.5µg IVX+
1µg IVX+
0.25µg IVX+
0µg IVX
50µg F1-V
20µg F1-V
5µg F1-V
20µg F1-V i.m.

FIG. 1

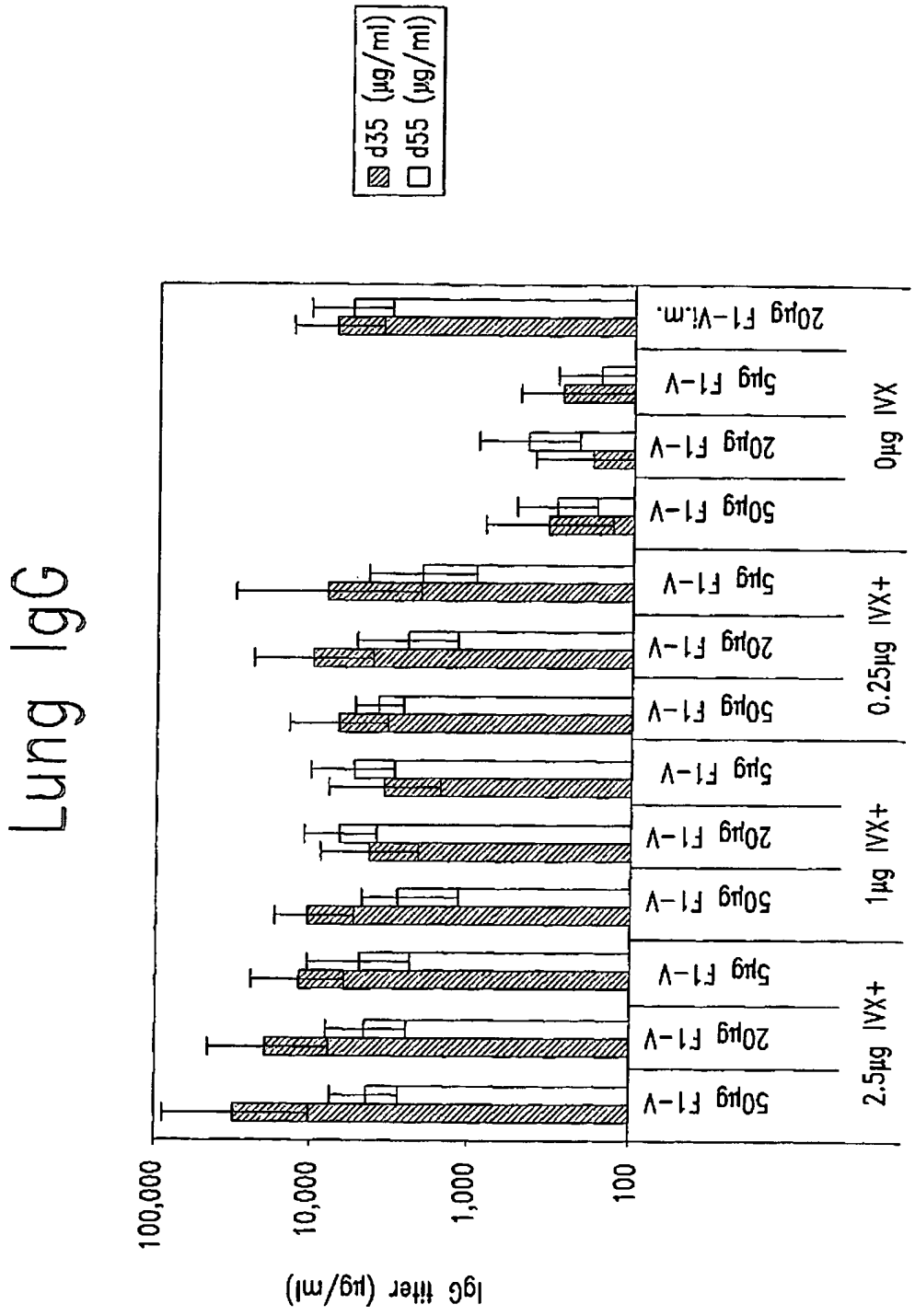
Lung IgG
d35 (µg/ml)
d55 (µg/ml)
log titer (µg/ml)
100,000
10,000
1,000
100
2.5µg IVX+
50µg F1-V
20µg F1-V
5µg F1-V
1µg IVX+
50µg F1-V
20µg F1-V
5µg F1-V
0.25µg IVX+
50µg F1-V
20µg F1-V
5µg F1-V
0µg IVX
50µg F1-V
20µg F1-V
5µg F1-V
20µg F1-V i.m.

FIG. 2

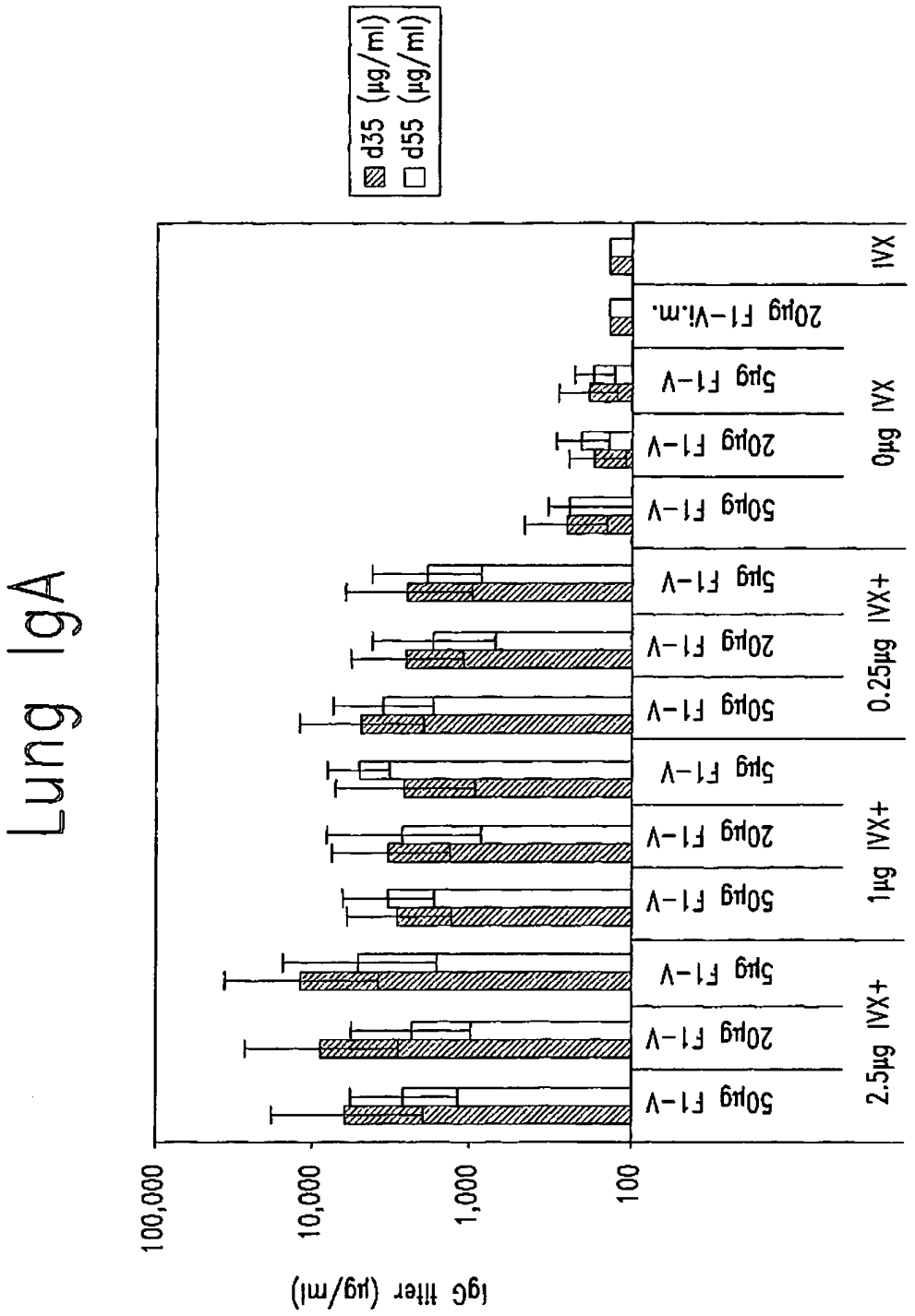
Lung IgA
IgG titer (µg/ml)
100,000
10,000
1,000
100
d35 (µg/ml)
d55 (µg/ml)
2.5µg IVX+
1µg IVX+
0.25µg IVX+
0µg IVX
IVX
50µg F1-V
20µg F1-V
5µg F1-V
20µg F1-V i.m.

FIG. 3

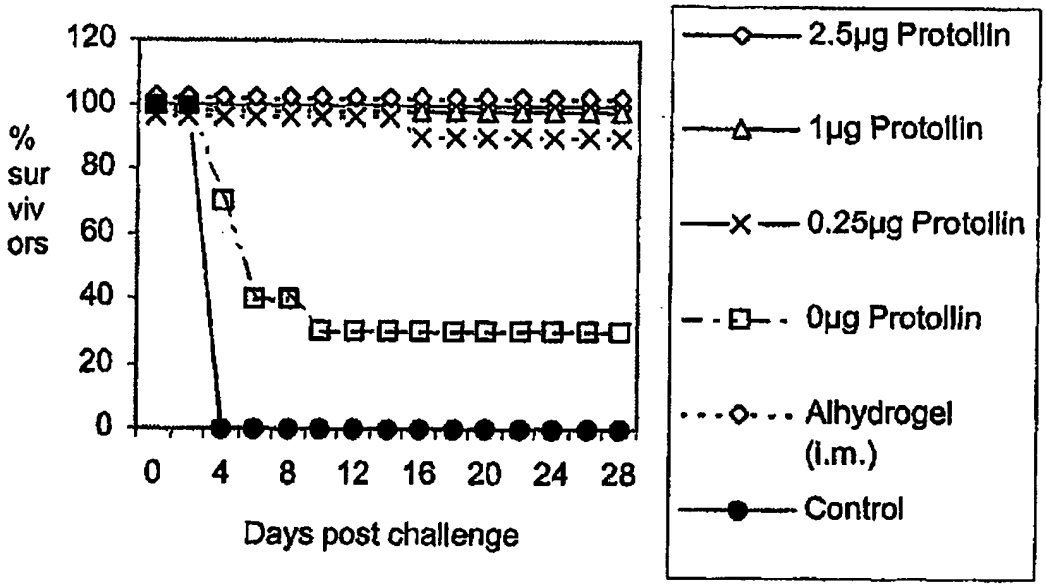
120
100
80
60
40
20
0
% sur viv ors
0 4 8 12 16 20 24 28
Days post challenge
2.5µg Protollin
1µg Protollin
0.25µg Protollin
0µg Protollin
Alhydrogel (i.m.)
Control

FIG. 4A

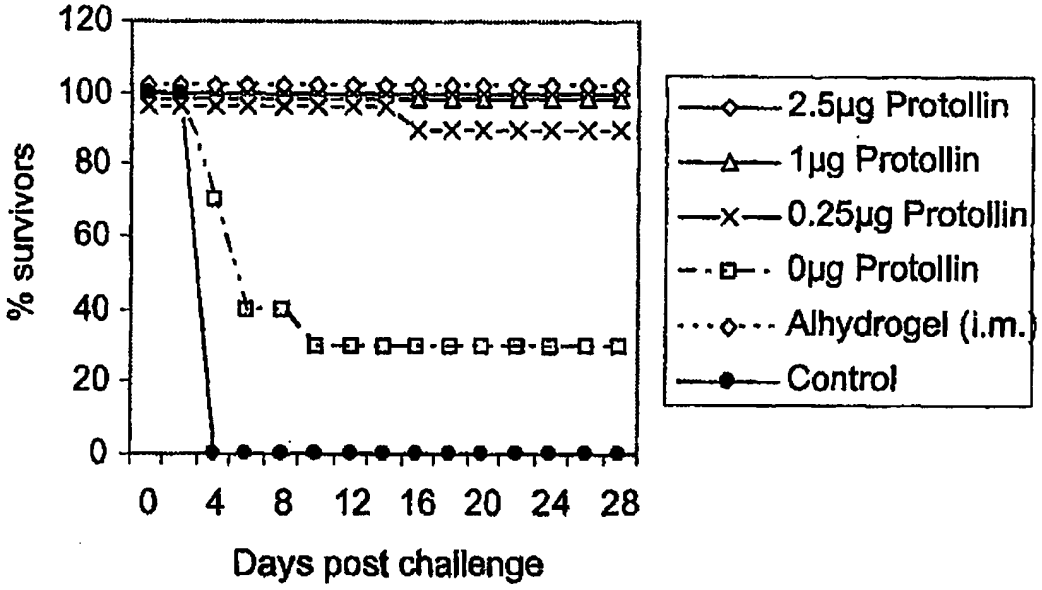
120
100
80
60
40
20
0
% survivors
0
4
8
12
16
20
24
28
Days post challenge
2.5µg Protollin
1µg Protollin
0.25µg Protollin
0µg Protollin
Alhydrogel (i.m.)
Control

FIG. 4B

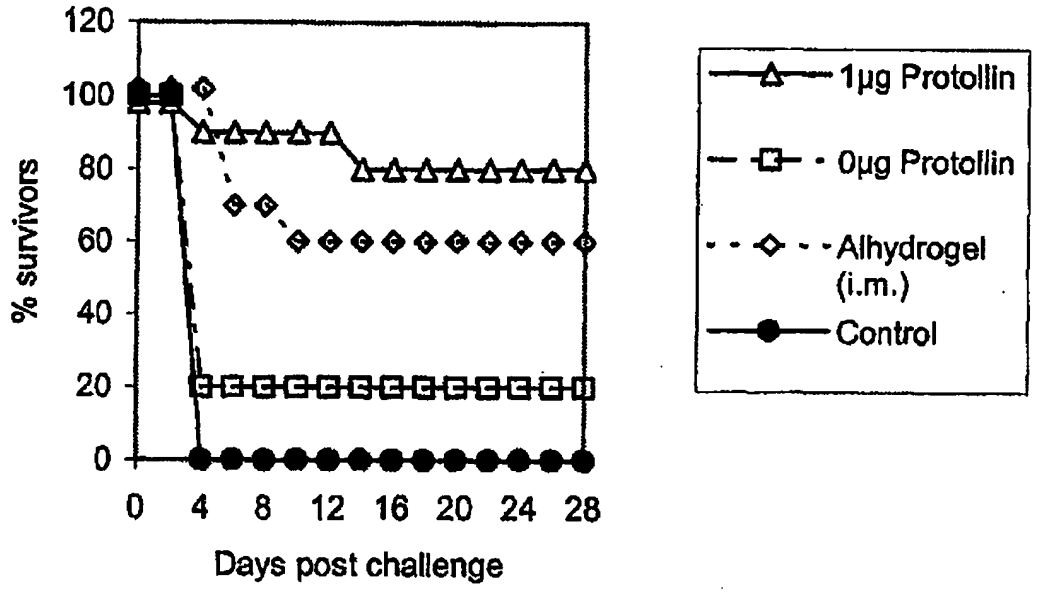
120
100
80
60
40
20
0
% survivors
0
4
8
12
16
20
24
28
Days post challenge
1µg Protollin
0µg Protollin
Alhydrogel (i.m.)
Control

FIG. 5A

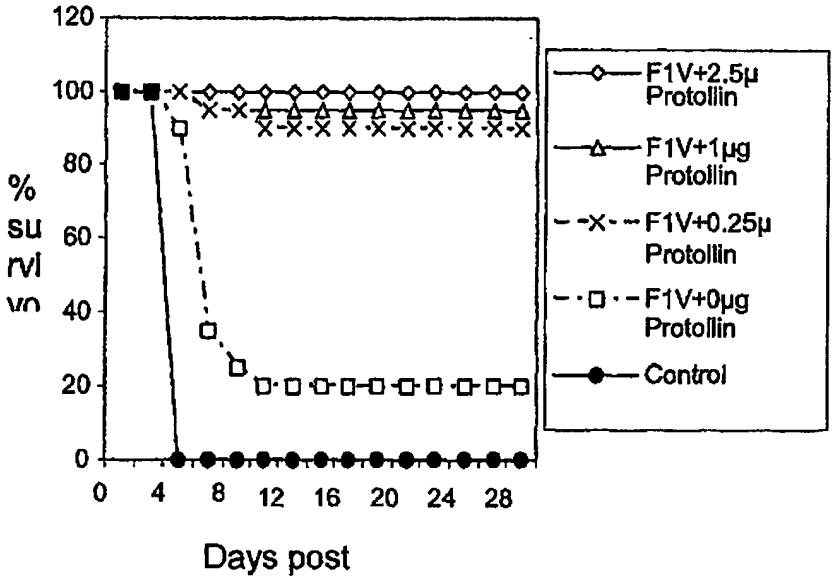
120
100
80
60
40
20
0
% survivor
0
4
8
12
16
20
24
28
Days post
F1V+2.5µ Protollin
F1V+1µg Protollin
F1V+0.25µ Protollin
F1V+0µg Protollin
Control

FIG. 5B

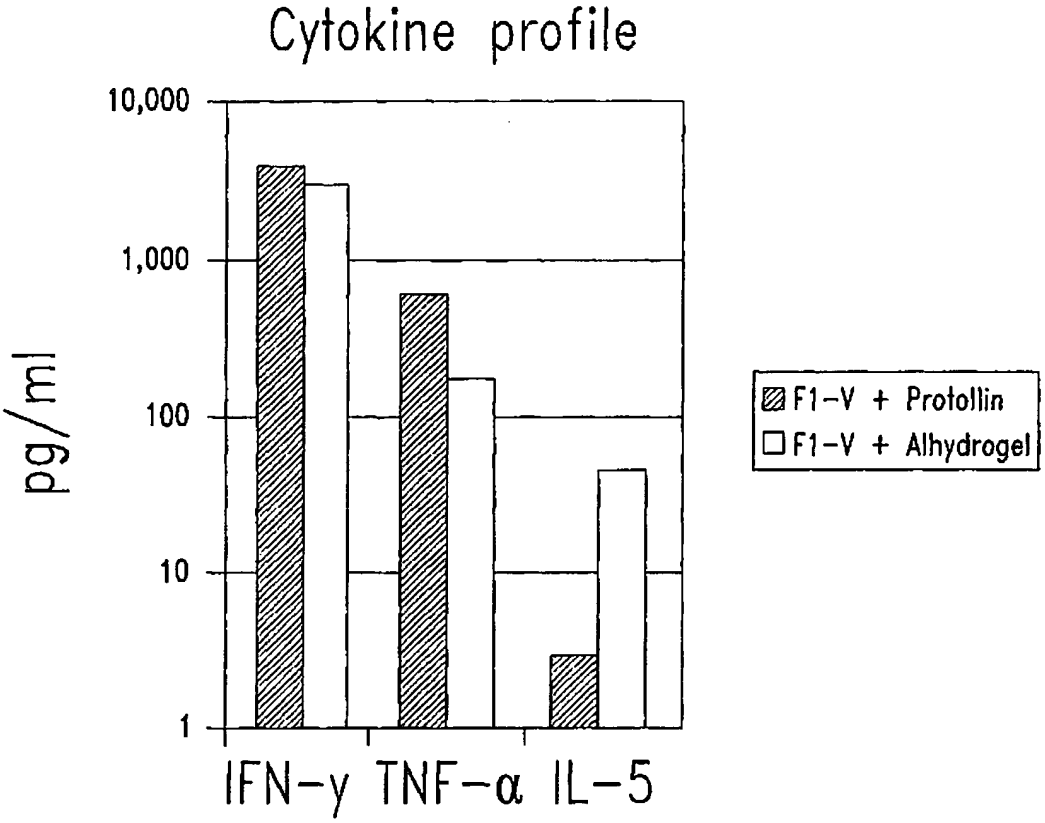
Cytokine profile
10,000
1,000
100
10
1
pg/ml
IFN-γ TNF-α IL-5
F1-V + Protollin
F1-V + Alhydrogel

FIG. 6

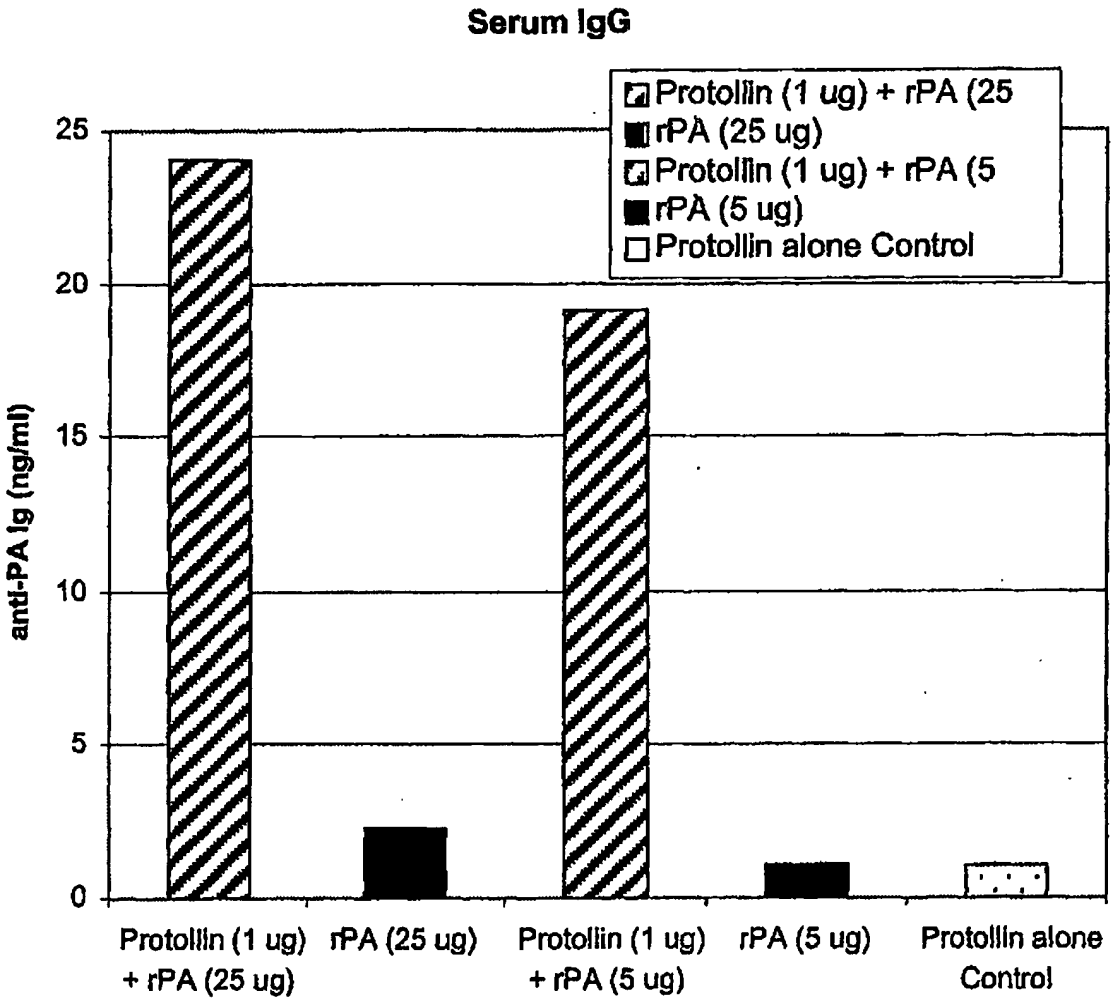
Serum IgG
Protollin (1 ug) + rPA (25
rPA (25 ug)
Protollin (1 ug) + rPA (5
rPA (5 ug)
Protollin alone Control
anti-PA Ig (ng/ml)
25
20
15
10
5
0
Protollin (1 ug) + rPA (25 ug)
rPA (25 ug)
Protollin (1 ug) + rPA (5 ug)
rPA (5 ug)
Protollin alone Control

FIG. 7A

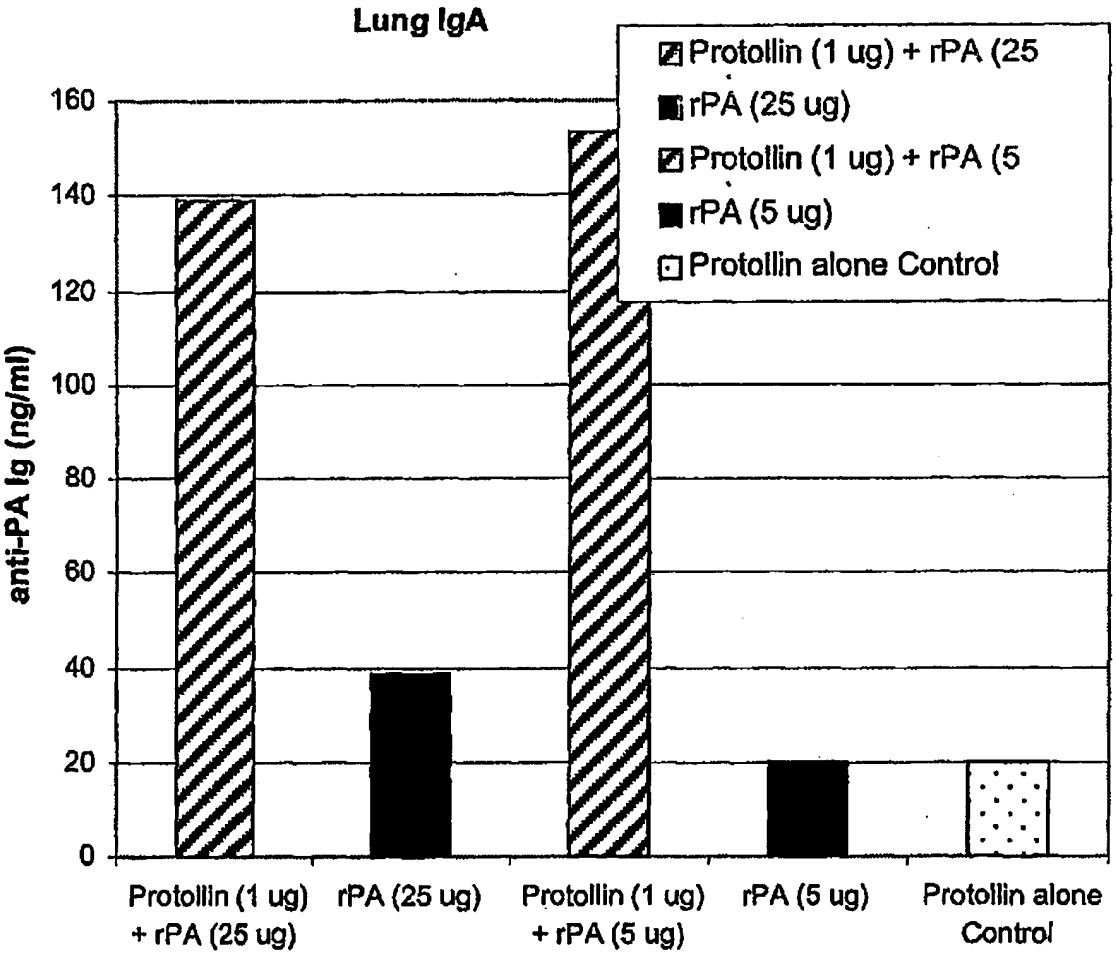
Lung IgA
Protollin (1 ug) + rPA (25
rPA (25 ug)
Protollin (1 ug) + rPA (5
rPA (5 ug)
Protollin alone Control
anti-PA Ig (ng/ml)
160
140
120
100
80
60
40
20
0
Protollin (1 ug) + rPA (25 ug)
rPA (25 ug)
Protollin (1 ug) + rPA (5 ug)
rPA (5 ug)
Protollin alone Control

FIG. 7B

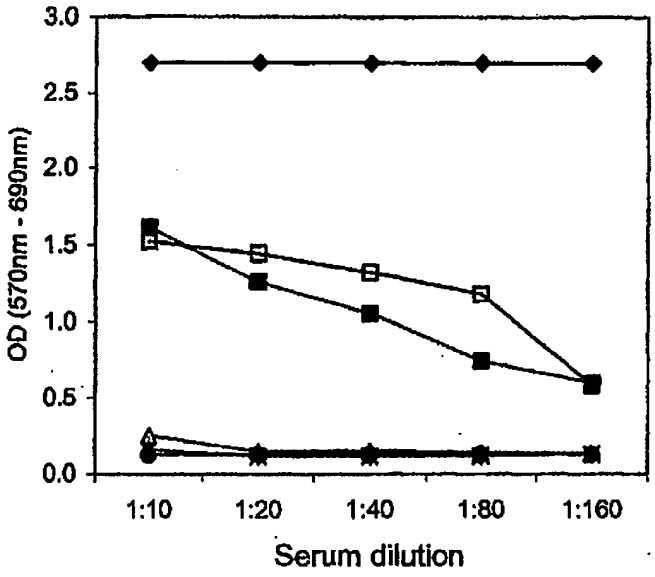
3.0
2.5
2.0
1.5
1.0
0.5
0.0
OD (570nm - 690nm)
1:10
1:20
1:40
1:80
1:160
Serum dilution

FIG. 8A

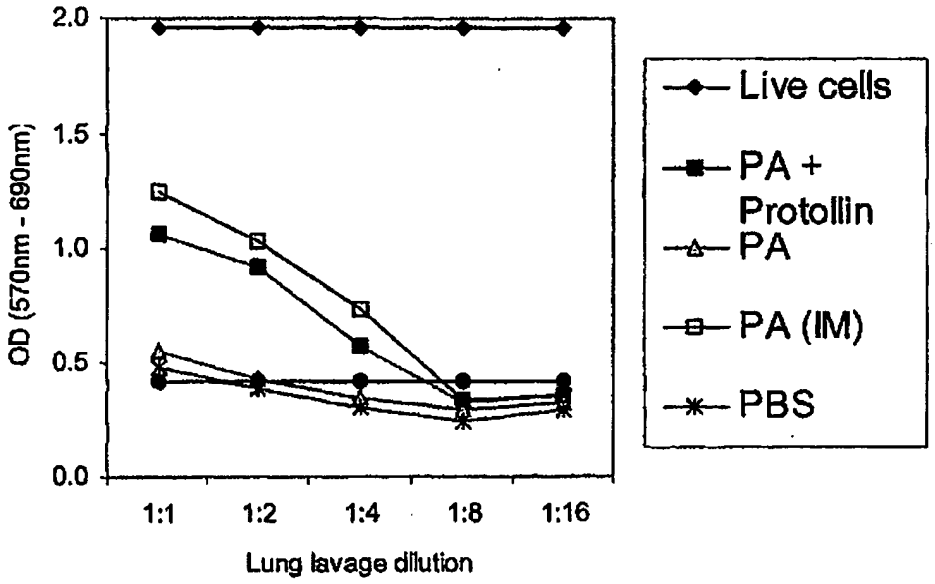
2.0
1.5
1.0
0.5
0.0
OD (570nm - 690nm)
1:1
1:2
1:4
1:8
1:16
Lung lavage dilution
Live cells
PA + Protollin
PA
PA (IM)
PBS

FIG. 8B

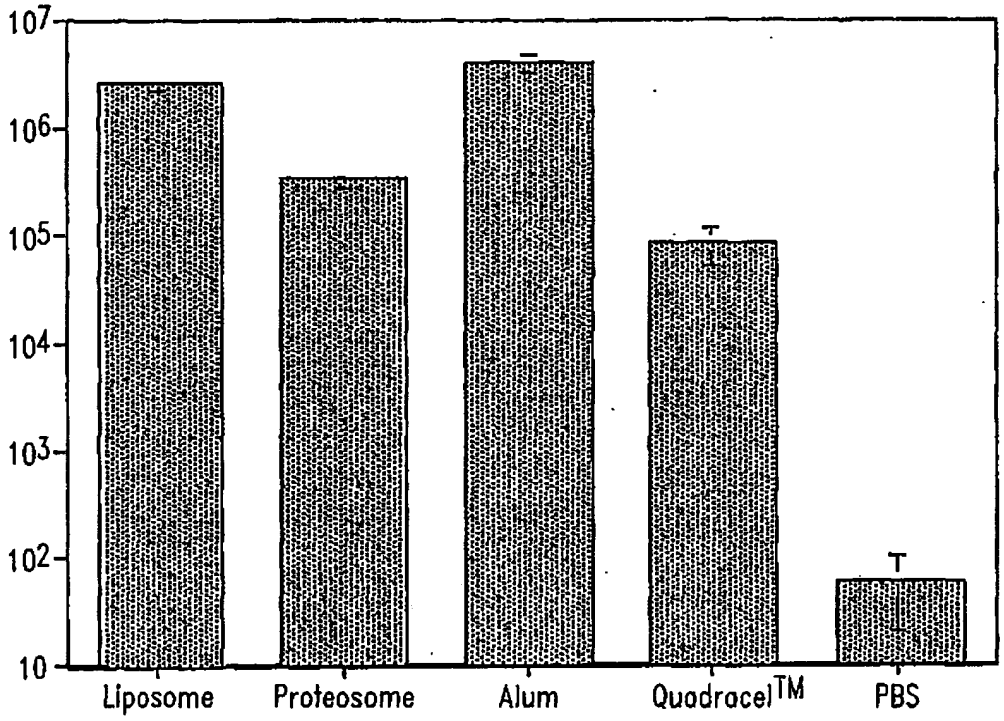
10^7
10^6
10^5
10^4
10^3
10^2
10
Liposome
Proteosome
Alum
Quadrocel™
PBS

FIG. 9

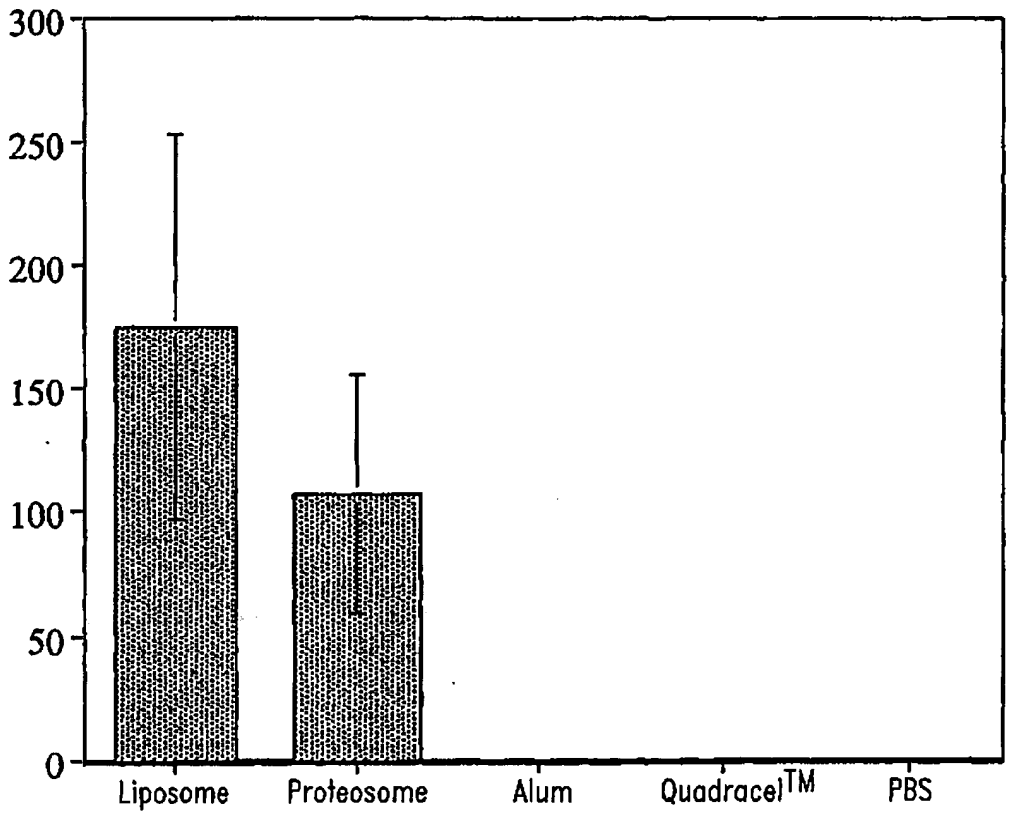
300
250
200
150
100
50
0
Liposome
Proteosome
Alum
Quadracel™
PBS

FIG. 10

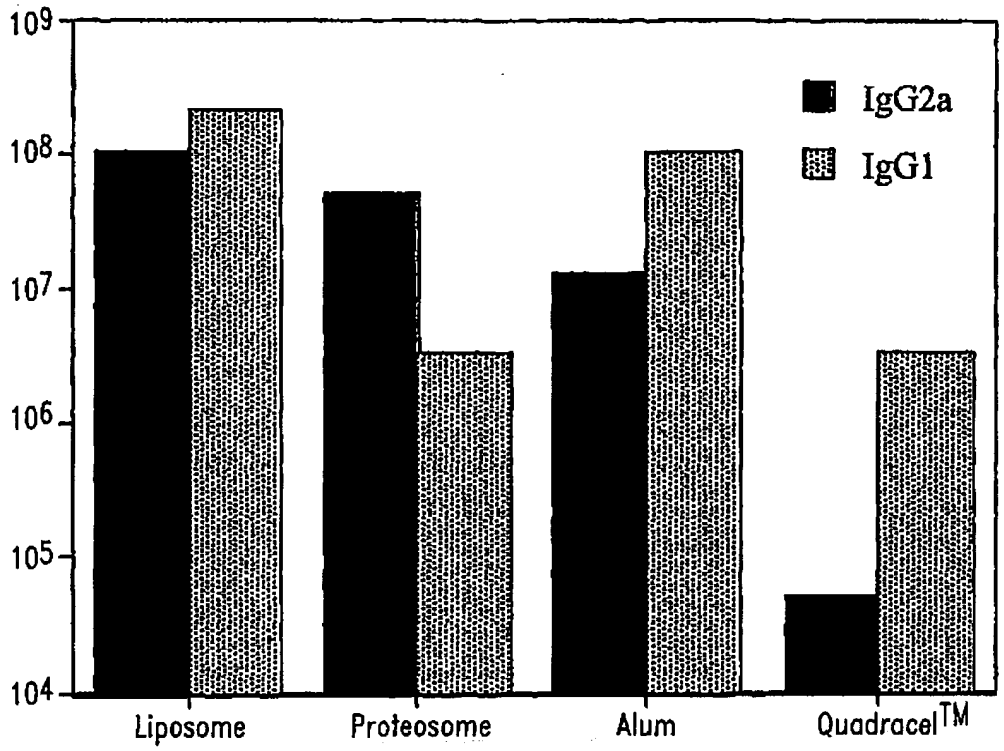
10^9
10^8
10^7
10^6
10^5
10^4
IgG2a
IgG1
Liposome
Proteosome
Alum
Quadracel™

FIG. 11

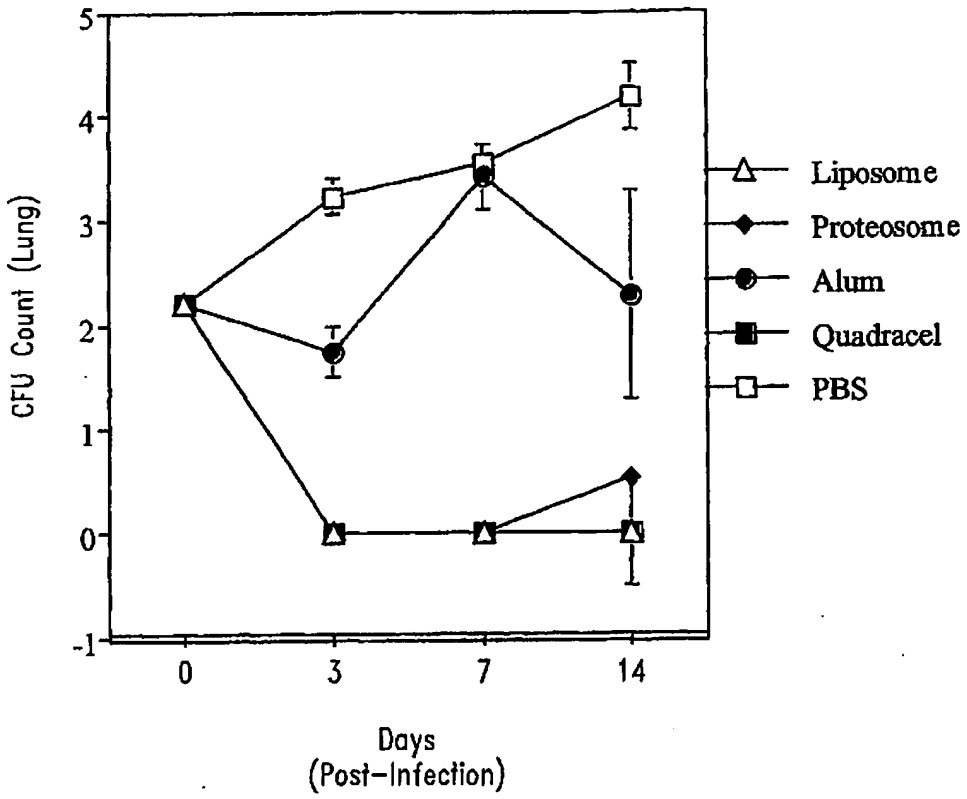
5
4
3
2
1
0
-1
CFU Count (Lung)
0
3
7
14
Days
(Post-Infection)
Liposome
Proteosome
Alum
Quadracel
PBS

FIG. 12

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